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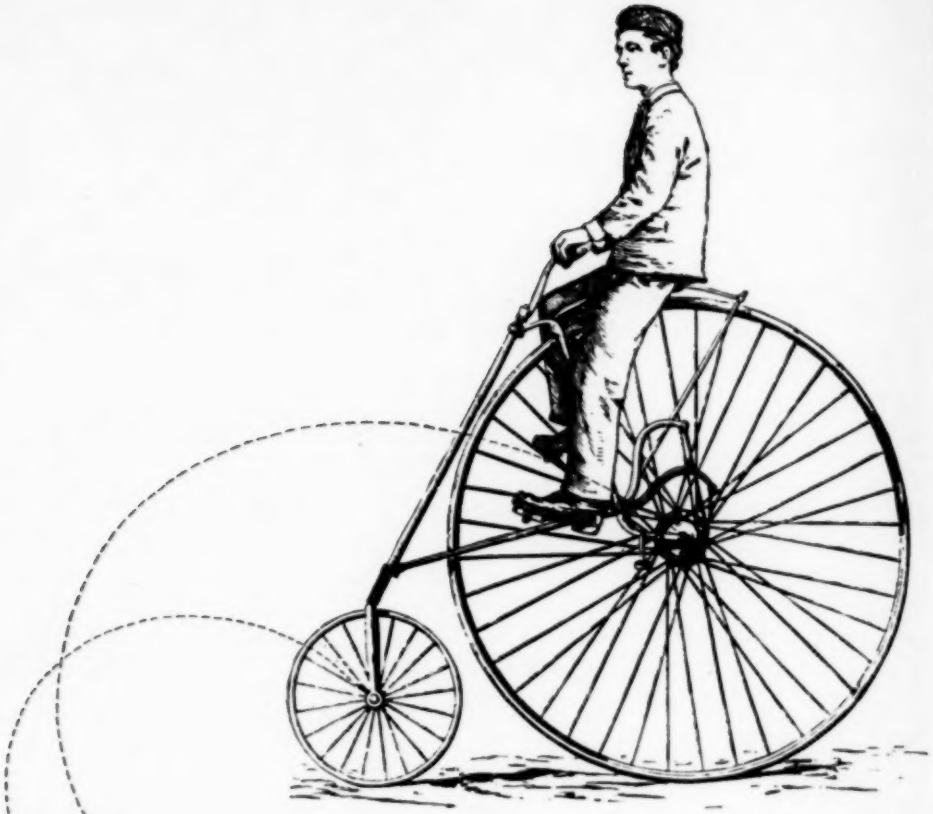

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JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION

JUSTIN L. POWERS, EDITOR, WASHINGTON, D. C.

VOLUME XXXVII — *FEBRUARY, 1948* — NUMBER 2 — CONSECUTIVE NO. 4

Some Unsolved Problems in the Chemistry of the Nucleic Acids*†

By R. STUART TIPSON

The nucleic acids are of immense importance in the chemistry of life-processes since they are found in the nucleus and cytoplasm of every cell. A brief summary of those details of their chemical structure which are known with certainty is presented; those which are still unknown or based purely on analogy are pointed out, and some suggestions for further research are made.

SUITABLY isolated, the molecules of nucleic acids are normally of exceedingly high molecular weight. It appears that the macromolecules of any one kind of nucleic acid may vary in size according to their source. The size is certainly dependent on the treatment to which the material is subjected during its isolation (1); this may involve partial depolymerization, dephosphorylation, or other degradation. The matter of the actual size of these various macromolecules will not be discussed here, except to say that the average molecular weight of the macromolecule of one nucleic acid examined may be of the order of half a million to a million (2); of another (3), about 37,000; of another (4), about 16,000 to 23,000.

We still know amazingly little concerning the manner in which these macromolecules are built up but there seems to be no doubt that a macromolecule is composed of a large number of mononucleotides. One way, but not the only possible way, of interpreting some of the results obtained is as follows:

Nucleic Acid Macromolecule
↓ (depolymerization)
Fundamental Polynucleotide Unit
↓ (hydrolysis)
2 purine bases (1 mole each)
+2 pyrimidine bases (1 mole each)
+a sugar (4 moles)
+phosphoric acid (4 moles)

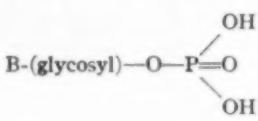
There are a number of reasons for believ-

* Received Aug. 5, 1947, from the Department of Research in Pure Chemistry, Mellon Institute, Pittsburgh 13, Pa.

† Based on a paper presented at the meeting of the Division of Biological Chemistry, American Chemical Society, held in Atlantic City, N. J., on April 15, 1947; revised since then in the light of certain recent discoveries. Parts of this discussion had already been published in *Advances in Carbohydrate Chemistry*, Academic Press, Inc., Vol. I, 1945, pp. 193–245.

ing that the hypothetical "fundamental polynucleotide unit" may exist, but this has not been proved beyond doubt. On the other hand, there appear to be, as yet, no reasons for believing that it does not exist. Claims that the fundamental unit is set free by enzymic (5) and chemical (6) depolymerization need substantiation. If the material is isolable it should be characterized carefully, because there is no proof that its constituent parts are always combined in the same way, in an invariant order. Furthermore, there appears to be no proof that any one macromolecule is an exact multiple of the supposed polynucleotide unit. It seems that some information on this point might be gained by taking various samples of a selected nucleic acid, each sample representing one molecular size (or as near as possible to it), and hydrolyzing each in order to see if the proportions of hydrolysis products are the *same* for each sample. Finally, if there is such a polynucleotide unit which, by repeated self-combination, yields the macromolecule, there is as yet no assurance that the inter-polynucleotide union is uniform throughout the macromolecule.

However, as regards the simple organic chemistry of the nucleic acids, these questions are of no immediate importance. We shall assume, as a working hypothesis, that any one macromolecule is a polymer of a fundamental polynucleotide molecule which may be defined chemically if we know (*a*) the number of its constituent mononucleotides, (*b*) the precise details of structure of each mononucleotide, and (*c*) the way and sequence in which the mononucleotides are united to give the fundamental polynucleotide. The mononucleotides are of two types, phosphoglycosyl-purines and phosphoglycosyl-pyrimidines, respectively:



where B = a purine or pyrimidine residue. Complete structural knowledge of a mononucleotide therefore involves determination of (*a*) the nature of the base; (*b*) the nature of the sugar; (*c*) the ring structure of the sugar residue; (*d*) the stereochemical disposition (α- or β-) of the sugar-base link; (*e*) the position of union of the sugar to the base; and (*f*) the position of the phosphoryl group on the sugar chain.

It is generally accepted that there are two main types of nucleic acid, similarly constituted but differing in certain components. Since the striking difference in the chemical and physical properties of these two acids is occasioned by the properties of their sugar components, they are now known as pentose-nucleic acid and desoxypentose-nucleic acid, respectively. There have been indications from time to time that other nucleic acids exist, but only these two types have been adequately characterized from the point of view of the organic chemist.

PENTOSENUCLEIC ACID

Let us consider the pentose-nucleic acid isolable from yeast. The bases were identified long ago as the purines adenine and guanine, and the pyrimidines uracil and cytosine. The four corresponding mononucleotides are adenylic acid, guanylic acid, uridylic acid, and cytidylic acid. We now know all the details of structure of the nucleotides. In the phosphopentosylpurines, the sugar is *D*-ribose (7), the configuration is β- (8), the sugar is attached at position 9′ of the purine (9), the sugar ring-structure is furanose (10), and the phospho group is situated at position 3 of the sugar residue (11). In 1946, Davoll, Lythgoe, and Todd (8) proved that adenosine has the β-configuration; since it is hydrolyzed by the same enzyme, guanosine probably has the same configuration.

Hence the pentosylpurine nucleotides are 3-phospho-9′-β-*D*-ribofuranosylpurines. They may be depicted as shown in Fig. 1.

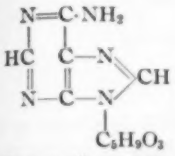
In the phosphopentosylpyrimidines, the sugar is *D*-ribose (12), the configuration is β- (8), the sugar is attached at position 3′ of the pyrimidine (13), the sugar ring-structure is furanose (14), and the phospho group is situated at position 3 of the sugar residue (15).

Hence the pentosylpyrimidine nucleotides are 3-phospho - 3′ - β - *D* - ribofuranosylpyrimidines. They have the formulas illustrated in Fig. 2.

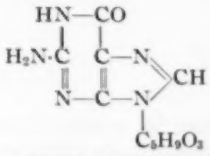
DESOXYPENTOSENUCLEIC ACID

The nitrogenous bases of desoxypentosenucleic acid are adenine, guanine, cytosine, and thymine (5-methyluracil). Levene discovered (16) that the sugar of the purine nucleosides is 2-desoxy-*D*-ribose. By ultraviolet absorption spectra studies, it has been shown to be attached at position 9 of the purines

(17), but its ring-structure has not been proved, and it is unknown whether these nucleosides are of α- or β-stereochemical configuration.

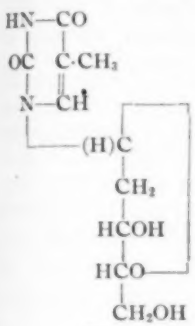


Desoxyribosyl-adenine

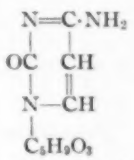


Desoxyribosyl-guanine

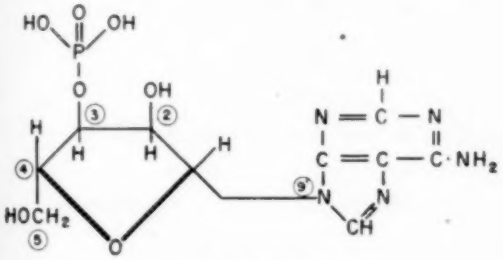
To date, there appears to be no proof at all that the sugar of the two desoxypentosyl pyrimidines is 2-desoxy-*D*-ribose, although there are indications that it is a 2-desoxypentose. From theoretical considerations of a chemical nature, it is almost certainly attached at position 3′ of the pyrimidine bases, but there is no positive proof of this. Evidence has been adduced indicating that the ring-structure of the sugar in thymidine is furanose (18), but that of the sugar in the cytosine nucleoside is unknown. It is not known whether these nucleosides are of α- or β-stereochemical configuration.



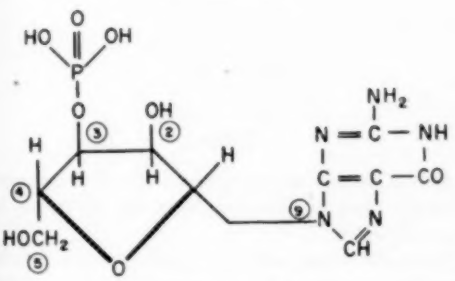
Thymidine



Desoxypentosyl-cytosine

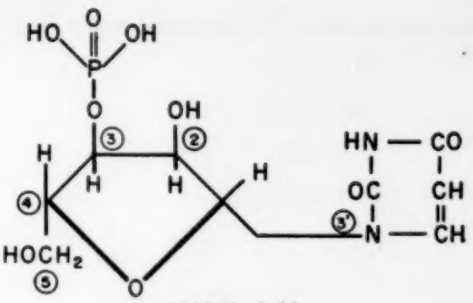


Adenylic Acid

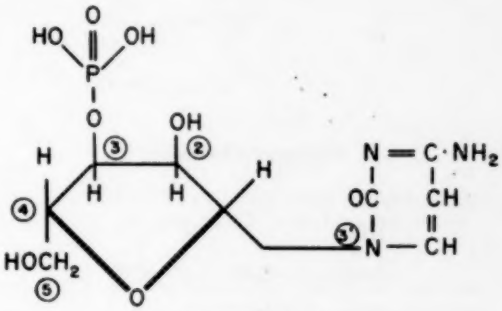


Guanylic Acid

Figure 1



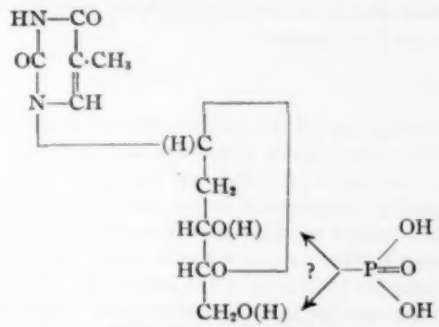
Uridylic Acid



Cytidylic Acid

Figure 2

Turning now to the corresponding nucleotides, the position of attachment of the phosphoryl group on the sugar is unknown. It cannot be at position 2 and if, as appears likely, the desoxypentose residue is furanose, it cannot be at position 4. Hence, it is probably either at position 3 or position 5. These

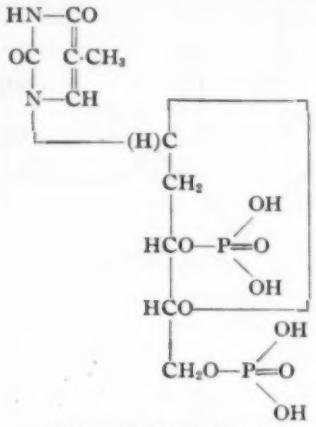


Monophospho-thymidine

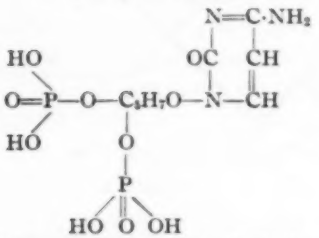
two pyrimidine nucleotides should be hydrogenated and then hydrolyzed with dilute mineral acid, and the resulting monophosphodesoxypentose studied. It will probably prove to be 3-phospho-2-desoxy-*D*-ribose, but this is still a matter of conjecture.

Now, in 1912, Levene and Jacobs (19) discovered that, on hydrolysis of the desoxypentosenucleic acid by means of boiling 2% sulfuric acid during two hours, diphosphoric esters of thymidine (20) and of the cytosine nucleoside (21) may be isolated. On catalytic hydrogenation, diphosphothymidine gave diphosphodihydrothymidine, a substance readily hydrolyzed by mineral acid to dihydrothymine and a reducing diphospho-sugar. Since thymidine is a

furanose derivative, the phosphoryl groups must be



Diphosphothymidine



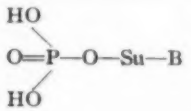
Diphospho-desoxypentosylcytosine

situated at positions 3 and 5 of the sugar. The diphospho-sugar should receive further study, since it offers the chance of identifying the sugar of these pyrimidine nucleotides; there is every reason to believe that it is 2-desoxy-*D*-ribose, but this has not yet been proved.

Having pointed out those features of structure of the mononucleotides which are still unknown, we may pass to the fundamental polynucleotide unit. Assuming that there *is* such a unit, the first point to be settled is the number of mononucleotides comprising it. Let us first consider *ribose-nucleic acid*.

Complete hydrolysis of the nucleic acid gives the four nitrogenous bases in equimolecular proportions (22). Mild alkaline hydrolysis of the nucleic acid gives directly an equimolecular mixture (23) of the four mononucleotides. Hence it appears reasonable to believe that the fundamental unit is a *tetra*nucleotide composed of adenylic acid, guanylic acid, uridylic acid, and cytidylic acid. It has been supposed that every such unit is identical with every other and that this unit consists of one molecule of each of the four nucleotides, always combined in the same relative order (sequence) and in the same way. Real proof of these speculations is still not at hand, but the available facts tend to favor the idea of a poly-tetranucleotide structure for yeast ribonucleic acid and for some desoxyribonucleic acids.

The next question which arises is, how, and in what order, are these four mononucleotides united. Combination between any two individual mononucleotides having the general formula



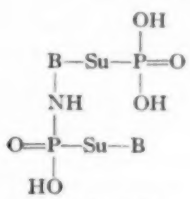
where Su = sugar residue, and B = purine or pyrimidine base, might be visualized as taking place in at least four different ways:

(*a*) through a phosphoamide link, the phosphoryl group of one nucleotide uniting with the amino group of the other (adenylic, guanylic, or cytidylic acid);

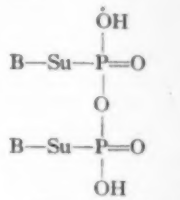
(*b*) through a pyrophosphate link, one phosphoryl group being combined with that of the other nucleotide;

(*c*) through an ether link, from a hydroxyl group of one ribose residue to one of the other ribose residue; or

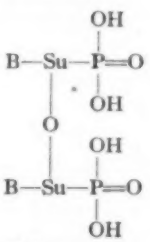
(*d*) through an ester link, the phosphoryl group of one nucleotide uniting with a hydroxyl group of the ribose residue of the other nucleotide, or with a lactim hydroxyl of one of the nitrogenous bases.



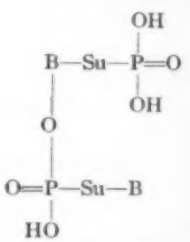
Phosphoamide



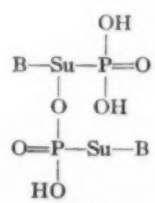
Pyrophosphate



Ether



Lactim-ester

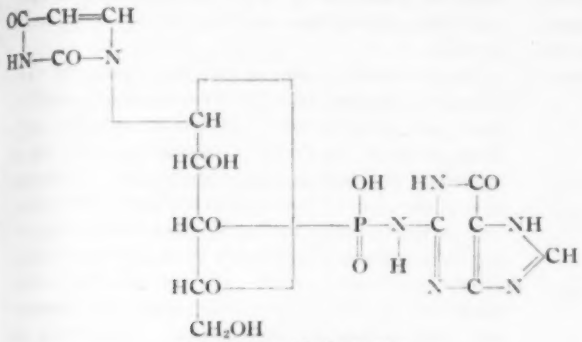


Diester

At one time or another, every one of these modes of union (and various combinations of them) has been advocated.

Since the three primary amino groups in ribosenucleic acid can be determined (24) by van Slyke's method, the possibility of phospho-amide linkages may be dismissed. In 1936, Bredereck (25) hy-

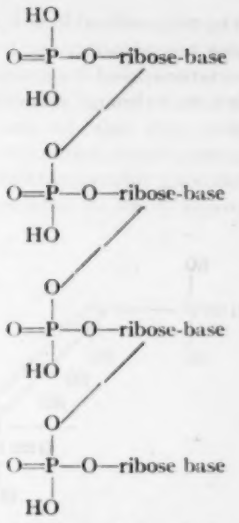
drolyzed some Boehringer ribosenucleic acid and isolated a very small amount of a material which he thought was "guanine-uridylic" acid of the formula



"Guanine-uridylic Acid"

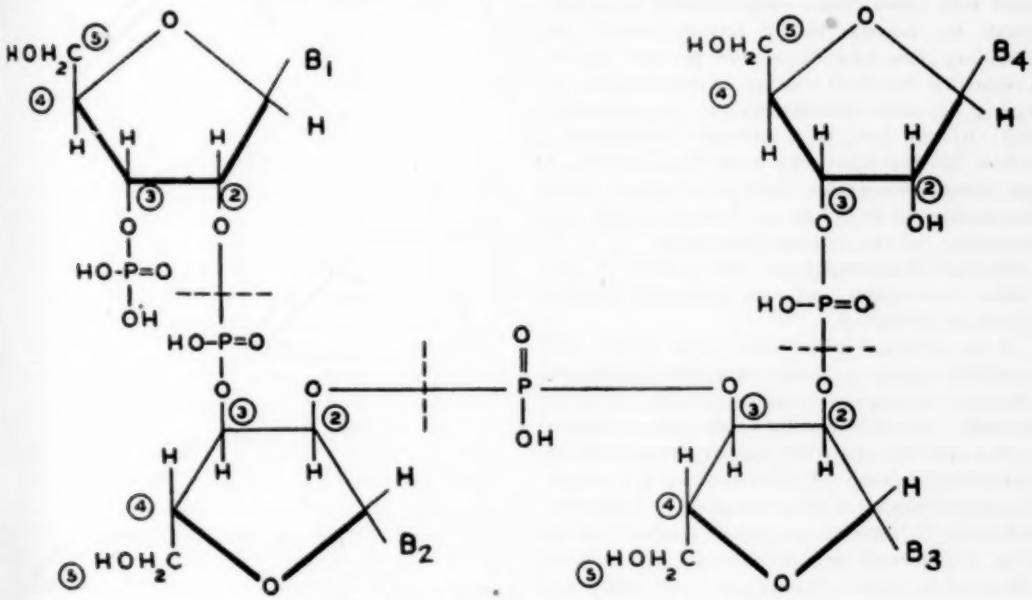
but it is now believed that this compound is an artifact, formed during the hydrolysis.

For various reasons, formulas involving pyrophosphate, ether, or lactim-ester linkages have been discarded. The formula at the right, proposed (26) by Levene in 1921, still seems the most likely. It depicts the fundamental polynucleotide as a tetranucleotide in which there are three di-ester phosphoric linkages between the individual mononucleotide residues. It was then suggested (27) that the tetranucleotide unit may be more precisely depicted as shown in Fig. 3. In this formula, each of the three linking phosphoryl groups is shared by position 2 of one ribose residue and position 3 of the adjacent ribose residue, so that, on hydrolysis, the four 3-phosphonucleotides result. It will be noted that the four primary sugar hydroxyl groups and one secondary hydroxyl are unsubstituted. Hence, it is suggested that some light might be thrown on this problem by appropriate alkylation or acylation of the fundamental tetranucleotide with subsequent hydrolysis to one di-derivative of ribose and three mono-alkyl or monoacyl derivatives.



Levene's Phospho-diester Formula for Ribosenucleic Acid Tetranucleotide

Many attempts have been made to decide the kind and position of the phosphoryl linkages by study of the number of phosphoric acid dissociations. Perhaps the first problem is to isolate pure tetranucleotide for study; then various homogeneous polymers of known molecular size should be examined.



Tetranucleotide Unit of Ribosenucleic Acid ($C_{38}H_{49}O_{29}N_{15}P_4$)

(Adapted from Tipson, R. S., "Advances in Carbohydrate Chemistry," Academic Press, Inc., Vol. I, 1945, p. 222.)

Figure 3

The tetranucleotide of Levene's formula should display one secondary and four primary phosphoric dissociations; and if a number of such units are joined in a chain through secondary phosphoric acid groups, there will only be one singly-bound phosphoric group present (terminally). This is depicted in the following abbreviated formula, in which all hydroxyl groups shown are attached to phosphorus:

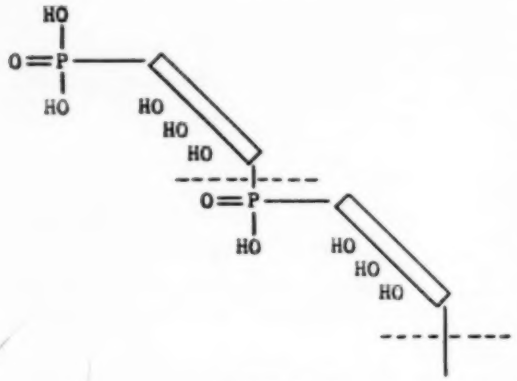


Figure 4

If the chain is long, it will be difficult to estimate the presence of this single group. Provided that the secondary phosphoric group of one tetranucleotide unit does not engage a phospho group of the adjacent tetranucleotide unit, the longer the chain, the closer will the number of acid dissociations approach four. According to Allen and Eiler (28), the polymerized acid does have four primary phosphoric dissociations per tetranucleotide unit, and, on treatment with ribonuclease (then believed to depolymerize the polymer to the tetranucleotide), one secondary phosphoric dissociation per unit appears. Levene (29) described a sample of ribosenucleic acid having 4.5 acid dissociations per tetranucleotide unit; it may have been partially depolymerized. Cohen (30) has shown that both ribosenucleic acid and desoxyribosenucleic acid may possess either four or five acidic groups per tetranucleotide unit, depending on the previous treatment. A careful correlation of molecular size with number of phosphoric dissociations and with hydrolysis products should be attempted.

If the secondary phosphoric group of one tetranucleotide engages a primary phosphoric group of the adjacent tetranucleotide unit, and this occurs repeatedly, Levene's formula would give a polymer having approximately three primary phosphoric dissociations. Levene (29) described such a sample; he considered that it probably possessed anhydride linkages. If intertetranucleotide linkage occurs (Fig. 5), through primary groups, the polymer will possess (approximately) one secondary and three primary dissociations per tetranucleotide unit. However, as shown in Fig. 6, it is perfectly possible that both types of intertetranucleotide linkage are present, namely, through secondary and through primary groups. There may even be cross-linkages from one chain to another. Alternatively, the polymer might consist of superimposed layers of a cyclic tetranucleotide. The possibility of hydrogen bonding should not be overlooked.

More recently, Gulland (31) has found, by electrometric titration, that yeast ribosenucleic acid has four acid dissociations per tetranucleotide unit, three of which are primary dissociations and one a secondary dissociation of phosphoric acid. Diminution of the molecular weight of the polytetranucleotide by mild alkaline hydrolysis gave results suggesting that a further secondary dissociation of phosphoric acid is set free, i.e., the fundamental tetranucleotide should be pentabasic, with three primary and two secondary dissociations. According to Gulland's results, possible formulas for the tetranucleotide (which polymerizes at A or B) are as shown in the formulas on the opposite page. However, if polymerization involves groupings at A and B, or from A or B of one tetranucleotide unit to a different phospho group of another unit, these formulas would give the wrong number of dissociations for the polymer. In polymers of such a tetranucleotide, it would be possible to split off as

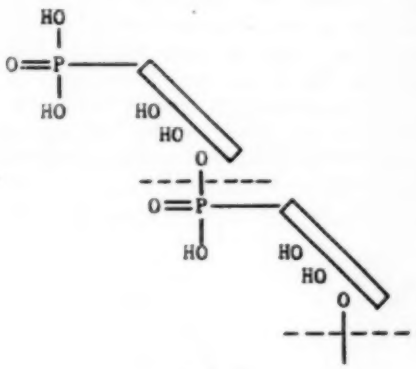


Figure 5

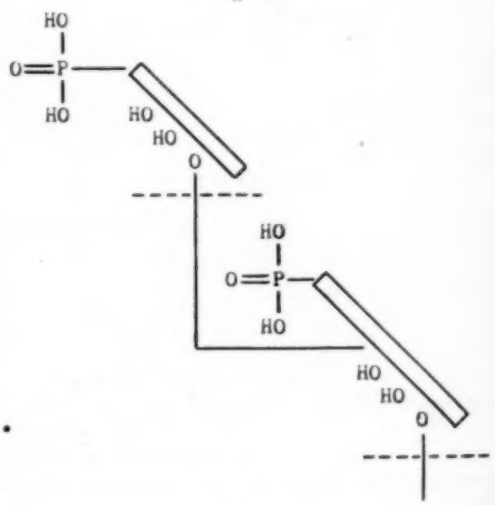


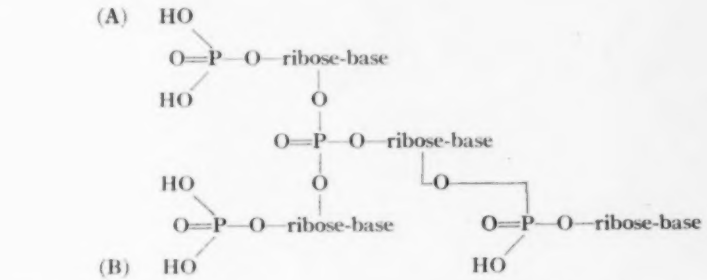
Figure 6

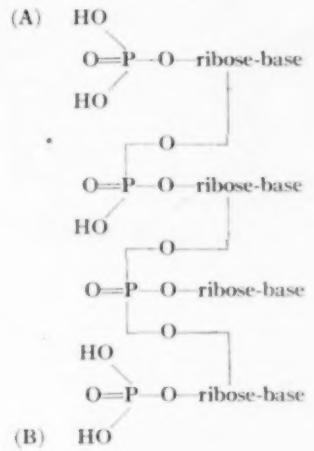
much as 25% of the total phosphorus without changing the main polynucleotide structure.

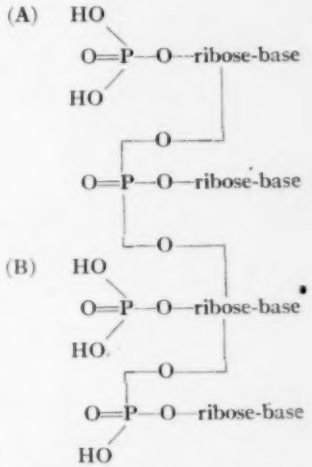
If some nucleotides were present as side chains, these might be removed (e.g., by ribonuclease) leaving a molecule still large enough to resemble the original acid in precipitability and in failure to dialyze. Indeed, Loring's work (32) on the action of ribonuclease on the polymer suggests that the "acid must contain at least two different types of linkages, one of which is labile and one of which is resistant" to the action of ribonuclease. Chemical hydrolysis (31) of the polymer apparently also causes fission of a link involving a secondary acidic group of a phosphoryl radical. It would be of interest to find out whether the products are identical, and whether the same phospho linkings are split in each type of degradation or depolymerization.

Finally, there is the question of the order in which the four nucleotides are united. Bredereck (33) has shown that mild hydrolysis of ribosenucleic acid with aqueous pyridine at 100° gives guanylic acid (G) plus a tri-nucleotide of adenylic acid (A), cytidylic acid (C), and uridylic acid (U). On further hydrolysis, A is split off. Bolomey and Allen (34) found that a certain nonspecific phosphatase preparation hydrolyzes ribosenucleic acid so that guanosine is, at first, liberated faster than adenosine. This suggests that guanylic acid is the first mononucleotide liberated, and adenylic acid the second. Hence, if dephosphorylation is not preceded by disruption to the *four* mononucleotides, guanylic acid is at one end of the chain, and adenylic acid is either next to it or at the other end. Possible formulas for the union of these are therefore:

G—U—C—A,
G—A—U—C,
G—A—C—U,
and G—C—U—A.







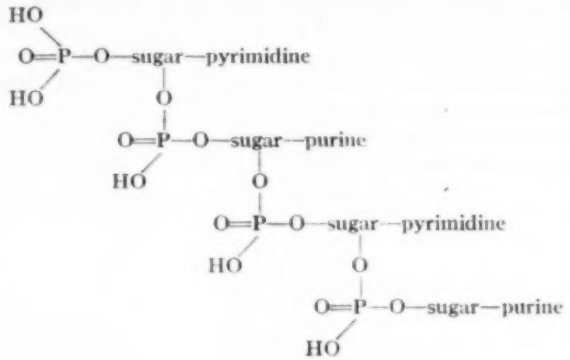
It would be interesting to arrest the hydrolysis when liberation of adenosine and guanosine approaches the maximum, and ascertain whether the pyrimidine nucleotides are present as a dinucleotide or as the two mononucleotides.

Turning now to the fundamental polynucleotide unit of *desoxyribosenucleic acid*, if such there be, the first point to be settled is the number and kind of mononucleotides comprising it.

Levene (35) showed that the two purine and the two pyrimidine bases occur in the acid in equimolecular proportions. Enzymic hydrolysis (36) gives all four nucleotides. Hence it appears reasonable to believe that the fundamental unit is a *tetra*nucleotide.

Levene (29, 37) and Bredereck (38) find that the acid, presumably depolymerized, has one secondary

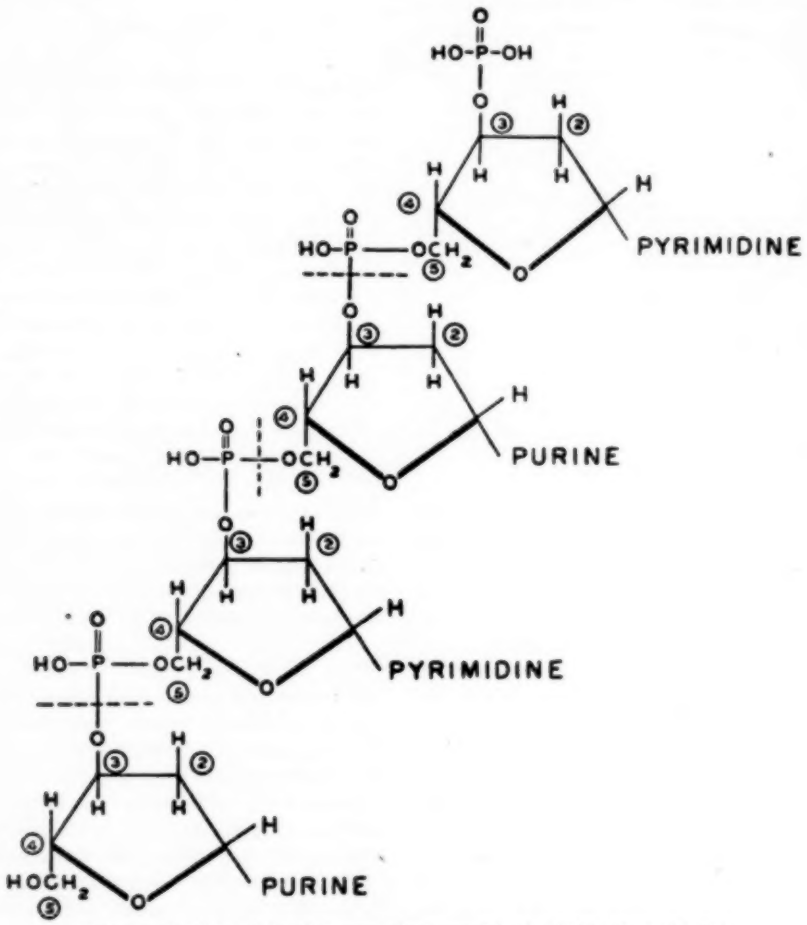
and four primary phosphoric dissociations, and since on suitable hydrolysis (19) it gives two diphosphodesoxypentosylpyrimidines, it has alternating purine and pyrimidine residues as follows:



Accepting the furanose structure for thymidine, and assuming it for the other three nucleosides, the phospho groups must join positions 3 to 5, as shown (27) in Figure 7 below.

On deamination it retains its tetranucleotide structure and pentabasicity (33), and hence contains no phosphoamide links. Evidence has been adduced recently (39*a*) that, in the polymer, hydrogen bonding may occur between purine and pyrimidine hydroxyl groups and some of the amino groups.

The polymerized acid is said (28, 39, 40, 41) to possess four acidic groups per tetranucleotide unit.



Tetranucleotide Unit of Desoxyribosenucleic Acid ($C_{39}H_{51}O_{25}N_{15}P_4$)
(Adapted from Tipson, R. S., "Advances in Carbohydrate Chemistry," Academic Press, Inc., Vol. I, 1945, p. 243.)

Figure 7

but a correlation between average basicity and average molecular weight does not appear to have been made. Cohen's studies (30) show that the acid may contain either four or five acidic groups per tetranucleotide unit, depending on the previous treatment. Depolymerization, either chemically (6) or with a depolymerase (42), appears to give a pentabasic tetranucleotide.

Finally, there is the question of the order in which the four nucleotides are united. It has previously been mentioned that the purines and pyrimidines alternate. By dephosphorylation (42, 43) of the supposed simple tetranucleotide, the nucleosides liberated first were those of adenine and thymine (T). This suggests that these are at the end or ends of the tetranucleotide chain, for example, thus:

A—C—G—T, and A—T—G—C.

Formulas A — T — C — G and A — G — C — T do not have alternating purines and pyrimidines and so are eliminated. Further study along these lines might permit us to decide which represents the true structure.

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Use of *Bacillus Subtilis* in the Three-Hour Cylinder-Plate Assay for Penicillin*

By ROBERTSON PRATT, FRANK M. GOYAN, JEAN DUFRENOY, and LOUIS A. STRAIT with the Laboratory Assistance of TOINIE JUNTUNEN and VIRGINIA LAMB†

The method of penicillin assay involving physical development of seeded plates has been extended to include plates seeded with *Bacillus subtilis*. The optimum conditions for tests with this organism are described. Advantages of using *B. subtilis* instead of *S. aureus* in this procedure are that assays can be completed more rapidly with the former organism, and that, with optimum conditions, the demarcation between areas of inhibition and of noninhibition is sharper. This rapid assay method provides reliable estimates of total penicillin activity in mixtures of different penicillins but is not suitable for performing "differential" assays.

SEVERAL procedures for assaying penicillin that retain the advantages inherent in the cylinder-plate method, but that reduce the time required for testing a solution of unknown concentration from about sixteen hours to three hours or less have been reported (1, 2). Of the several methods described, the one in which the test plates are rendered analogous to photographic emulsions has proved most useful for routine assays. This technique, details of which have been published (1), was developed using *Staphylococcus aureus* as the test organism.

The technique is not limited to this organism, however, but may be applied satisfactorily, *mutatis mutandis*, to plates seeded with other bacteria. Since a rough form of *Bacillus subtilis* (NRRL strain No. B-558) is commonly employed in performing assays of mixtures of different penicillins, it seemed of interest to determine the optimum conditions for physical development of plates seeded with this organism and to ascertain whether satisfactory subtilis/aureus ratios can be secured by the rapid method. This ratio varies for different penicillins and has been used as an indication of the proportions of different penicillins present in mixtures (3, 4).

* Received Sept. 16, 1947, from the University of California College of Pharmacy, The Medical Center, San Francisco.

† Acknowledgment is made to the Cutter Laboratories, Berkeley, Calif., for a generous grant that made execution of this work possible.

EXPERIMENTS AND RESULTS

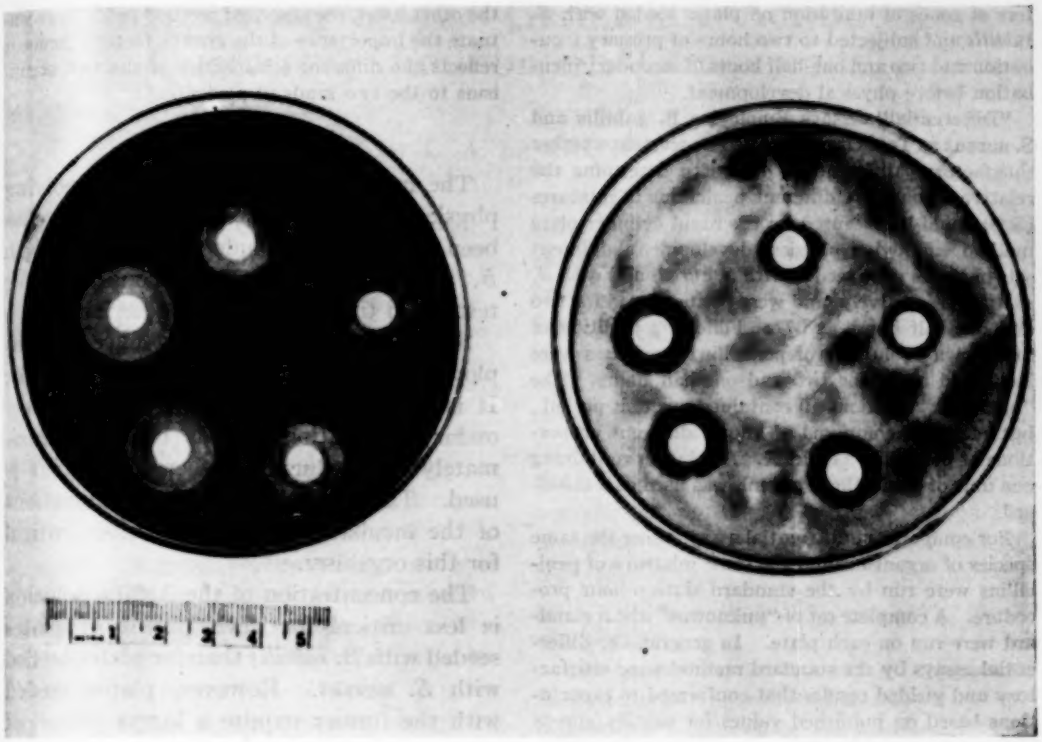
Physical Development of Plates Seeded with B. subtilis.—In general, the procedures to be used with *B. subtilis* are similar to those for *S. aureus*. They differ principally in the duration of the periods of incubation and of subsequent development. The incubation times for plates seeded with *B. subtilis* should be shorter, and should be more accurately controlled because duration of incubation is more critical with this organism than with *S. aureus*. It is important with *B. subtilis* that both primary and secondary incubation periods be relatively short and of approximately equal duration. When the ratio, primary incubation period/secondary incubation period, is markedly greater than 1.0, the plates are characterized by a uniform coarseness over the entire surface, and by small zones of inhibition which are of approximately equal diameter for all concentrations of penicillin from 0.5 to 8 units/ml. When the ratio is less than 0.8, development of the entire plate is very weak, and extremely faint zones are obtained. The zones, however, show the expected correlation between diameter and concentration of penicillin.

This is in sharp contrast to *S. aureus* for which the duration of the primary and secondary incubation periods is not so critical.

The concentration of $AgNO_3$ solution that is used is less critical for assays involving *B. subtilis* than for those with *S. aureus*. In general, for a given set of conditions of temperature, concentration of silver nitrate solution, and of exposure to silver nitrate and to light, a longer period of development is required for plates seeded with *B. subtilis*. The optimum conditions for developing plates seeded with the two different organisms are summarized in Table I.

The contrast between areas of inhibition and of noninhibition on plates seeded with *S. aureus* and subsequently subjected to a proper regimen of "physical development" is very pronounced, but the contrast between similar areas on plates seeded with *B. subtilis* is even more marked. Photographs of typical assay plates seeded with *B. subtilis* and with *S. aureus* and processed by physical development are reproduced in Fig. 1. Some definition has been lost in the processes of photography and reproduction.

Attention should be directed also to the different pattern that physical development reveals on assay



1A 1B

Fig. 1.—Reproduction of photographs of penicillin assay plates processed by impregnation with silver followed by exposure to light and subsequent physical development. Zones for solutions containing 1, 0.5, 2, 4, and 8 units/ml., respectively, are arranged in clockwise order beginning with those at the top. 1A (left), plate seeded with *S. aureus;* primary and secondary incubation periods each 3 hours. 1B (right), plate seeded with *B. subtilis;* primary and secondary incubation periods 2 hours and 2.5 hours, respectively.

TABLE I.—COMPARISON OF PHYSICAL DEVELOPMENT OF AGAR PLATES SEEDED WITH *S. aureus* AND WITH *B. subtilis* FOR ASSAY OF PENICILLIN

Condition	*S. aureus*	*B. subtilis*
$AgNO_3$		
Optimum concentration	0.1%	0.1–0.12%
Useful range of concentration	0.1–0.12%	0.1–0.16%
Primary incubation		
Optimum duration	3 hr.	2 hr.
Useful range	2.5–4 hr.	2–3 hr.
Secondary incubation with penicillin		
Optimum duration	3 hr.	2.5 hr.
Useful range	2.5–4 hr.	2–3 hr.
Time of development (at 22–25°)	7–10 min.	10–15 min.
Linear range of calibration curve (Log–Log)	1–8 units/ml.	1–8 units/ml.
Useful range of calibration curve	0.5–8 units/ml.	0.5–8 units/ml.

plates seeded with *B. subtilis* as compared with plates seeded with *S. aureus*. This can be seen in Fig. 1.

When primary and secondary incubation periods that give clearest development are chosen the calibration curves that are obtained by plotting log diameter of inhibition zone against log concentration of penicillin have approximately the same slope. The figures in Table II show average diame-

TABLE II

Diameter of Zone	Units of Penicillin/Ml. 0.5	1.0	2.0	4.0	8.0
	Mm.	Mm.	Mm.	Mm.	Mm.
Observed	12.9	15.75	16.60	18.10	19.00
Calculated[a]	...[b]	15.73	16.76	17.89	19.05

[a] Statistically calculated value based on an assumed linear log diameter/log concentration function.

[b] No calculated value given for this concentration, since this range is slightly off the linear curve.

ters of zones of inhibition on plates seeded with *B. subtilis* and subjected to two hours of primary incubation and two and one-half hours of secondary incubation before physical development.

"Differential" Assays Employing B. subtilis and S. aureus as Test Organisms.—To ascertain whether satisfactory "differential" assays to determine the relative amounts of different penicillins in mixtures (3, 4) could be executed by the rapid cylinder-plate method employing physical development, different sets of plates were seeded with *S. aureus* and with *B. subtilis*, respectively, and were preincubated for two and one-half hours at 37°. Following addition of "unknown" solutions of penicillin, the plates were incubated again for two and one-half hours. The "unknown" solutions all contained one unit per ml., but each was composed of known different proportions of penicillins G and X. A solution containing one unit of penicillin G per ml. was used as a standard.

For comparison, differential assays using the same species of organisms and the same mixtures of penicillins were run by the standard sixteen-hour procedure. A complete set of "unknowns" and a standard were run on each plate. In general, the differential assays by the standard method were satisfactory and yielded results that conformed to expectations based on published values for subtilis/aureus ratios determined by this method (3). The differential values determined by the short method involving physical development varied in a random manner, however, and indicated that this technique is not adaptable to the performance of such assays. This result was not unexpected, since the inhibition zones that develop in the cylinder-plate assays represent the net visible expression of opposing factors, *viz.*, physical factors represented by diffusion, concentration gradient, etc., and biological factors represented by the growth of the organism. In the short methods, the biological factor (growth) is reduced to a minimum and the effect of the physical factors is relatively accentuated. Since relatively little difference would be anticipated in the rates of diffusion of penicillins G and X, it is reasonable that such a method should fail to reveal significant or consistent differences in the differential assays. On the other hand, the standard method tends to accentuate the importance of the growth factor; hence it reflects the different sensitivities of the two organisms to the two kinds of penicillin.

SUMMARY

The method of penicillin assay involving physical development of seeded plates has been extended to include plates seeded with *B. subtilis*. The optimum conditions for tests with this organism are described.

Shorter incubation periods may be employed with *B. subtilis* than with *S. aureus*. It is important that the primary and secondary incubation periods be of approximately equal duration when *B. subtilis* is used. The absolute and relative durations of the incubation periods are more critical for this organism.

The concentration of the $AgNO_3$ solution is less critical for development of plates seeded with *B. subtilis* than for plates seeded with *S. aureus*. However, plates seeded with the former require a longer period of exposure to the developer solution following treatment with $AgNO_3$ and exposure to light.

The rapid physical development method, as it is now employed, is not applicable to the determination of subtilis/aureus ratios that give a reliable indication of the proportions of penicillins X and G in mixtures of the two. The reasons for this are pointed out.

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(4) Unpublished data given in private communication from Dr. Herman Beniams, Research Division, Cutter Laboratories, Berkeley, Calif.

Inactivation of the Germicidal Action of Quaternary Ammonium Compounds*

By C. A. LAWRENCE

The need for means of inactivating the antibacterial activity of quaternary ammonium germicides is summarized. Anionic detergents and soaps fail to inactivate surface-active cationic detergents. Suramin sodium is the most satisfactory inactivator of any in a group of sulfonates and sulfonic acids tested for this purpose.

It is of interest to note that following the introduction of effective chemotherapeutic or germicidal agents for the destruction of microorganisms, reports appear early in the literature on methods for the inactivation of the antibacterial compounds. These studies involve not only an attempt to explain the mechanism of action of the new derivatives, but also a means to determine the presence or numbers of living organisms that may remain dormant (inhibited) in dry powders or solutions of these products.

The present accepted sterility tests for liquids and solids require a medium containing thioglycollate (1, 2). The latter compound is used not only for the detection of aerobic and anaerobic microorganisms in a single procedure, but also for its ability to neutralize the bacteriostatic effect of mercurials that are added to many pharmaceuticals.

Additional examples of methods that are used for inactivating the antibacterial properties of various compounds may be found in the following combinations: sodium thiosulfate *versus* chlorine (3, 4); *p*-aminobenzoic acid *versus* sulfanilamide (5, 6); penicillinase *versus* penicillin (7, 8, 9); ferric chloride *versus* phenol (10); and glucose *versus* streptomycin (11).

With the increasing popularity that the quaternary ammonium germicides are receiving in the fields of medicinal and general disinfection, the need for a suitable means for inactivating the high germicidal activity of this group of compounds becomes apparent. In the inactivation of these germicides, as well as the antibacterial agents mentioned before, several important factors must be taken under consideration in selecting the most desirable means for accomplishing this. The neutralizing substance must, as completely as possible, inactivate the bactericidal and bacteriostatic properties of the compound and maintain this state over a prolonged period of time. The inactivating agent should, furthermore, have no antibacterial action itself and little, if any, growth-promoting properties upon microorganisms.

The quaternary ammonium germicides belong to a group of compounds known as "surface-active" agents or detergents. These may be classified as non-ionic, or ionic. The latter may be subdivided as (*a*) cationic, in which the lipophilic (oil-attracting) group becomes the cation (+); (*b*) anionic, in which the lipophilic group becomes the anion (−). The surface-active quaternary ammonium germicides ionize in water as cationic compounds in which the long chain part of the molecule is positively charged.

Cationic and anionic detergents will neutralize and often precipitate each other. Domagk (12), who called attention to the high antiseptic properties of the quaternary ammonium germicides, was first to indicate the neutralizing action of an anionic detergent (soap) upon a cationic detergent. Baker, Harrison, and Miller (13) noted the inhibiting action of the anionic compounds, decyl sulfate and sodium taurocholate, upon Benzalkonium Chloride (U. S. P. XIII). Of particular interest were the observations of Valko and DuBois (14) who noted that bacteria which are apparently "killed" by a cationic quaternary ammonium germicide may be revived by the addition of a high molecular anion, provided that application of the latter occurs within a time period of ten to thirty minutes.

* Received Oct. 3, 1947, from Winthrop Chemical Company, Inc., Rensselaer, N. Y.

TABLE I.—EFFECTS OF SEVERAL COMPOUNDS UPON ANTIBACTERIAL ACTION OF BENZALKONIUM CHLORIDE (1:11,000) AGAINST *S. aureus*—COLONIES PER 4-MM. LOOP

Test Agent	1 KCNS 15 Min. D[a]	1 KCNS 15 Min. C	1 KCNS 30 Min. D	1 KCNS 30 Min. C	2 $K_3Fe(CN)_6$ 15 Min. D	2 $K_3Fe(CN)_6$ 15 Min. C	2 $K_3Fe(CN)_6$ 30 Min. D	2 $K_3Fe(CN)_6$ 30 Min. C	3 Duponol C 15 Min. D	3 Duponol C 15 Min. C	3 Duponol C 30 Min. D	3 Duponol C 30 Min. C	4 Santomerse T. 15 Min. D	4 Santomerse T. 15 Min. C	4 Santomerse T. 30 Min. D	4 Santomerse T. 30 Min. C	5 Naphthalene (g) 15 Min. D	5 Naphthalene (g) 15 Min. C	5 Naphthalene (g) 30 Min. D	5 Naphthalene (g) 30 Min. C	6 Naphthalene (l) 15 Min. D	6 Naphthalene (l) 15 Min. C	6 Naphthalene (l) 30 Min. D	6 Naphthalene (l) 30 Min. C
1:200	0	186	0	163	0	17	0	30	2	4	1	4	0	0	0	0	13	0	4	0	39	36	19	21
1:400	0	230	0	201	0	52	0	46	1	1	0	1	0	0	0	0	6	18	8	3	22	49	18	36
1:800	0	156	0	222	0	47	0	47	4	0	3	0	0	0	0	0	0	27	0	5	11	40	7	13
1:1600	0	...	0	...	0	41	0	30	28	8	17	0	0	0	0	0	0	19	0	4	0	34	0	20
No Inactivator or Quaternary	...	182	...	213	...	58	...	52	...	54	..	49	...	62	...	76	...	104	...	57	...	24	...	44

[a] D = Quaternary ammonium detergent (1 cc. of 1:1000 aqueous) + inactivator (10 cc.) + organisms (0.1 cc.).
C = Control. Inactivating agent (10 cc.) + organisms (0.1 cc.).

Klein and Kardon (15), on the other hand, have recently demonstrated that "reversal" of quaternary ammonium action cannot be evidenced once the organisms have been exposed to germicidal concentrations of the compounds. Under certain conditions, the addition of an anionic detergent (sodium decyl sulfate, sodium lauryl sulfate, etc.) to the treated bacterial suspensions merely interrupts the continued action of the cationic detergents, thereby permitting surviving organisms to grow out.

Unlike the ionic reaction between anionic and cationic compounds is the interference of the action of quaternary ammonium germicides by phospholipids. Baker, *et al.* (13), observed the protective action of lecithin, cephalin, and sphingomyelin upon bacteria exposed to the detergents. However, in this instance also, the lipids are ineffective unless they are added to a suspension of organisms before or at the same time as the germicide.

METHOD I

Series I.—In the quantitative chemical method for determining the content of quaternary ammonium compounds in solutions, potassium ferricyanide (16) or potassium thiocyanate may be used to combine chemically with molar equivalents of the germicides. Conceivably, therefore, complete inactivation of the antibacterial action of the compounds may accompany combination of the potassium salts with them. A study was made of the effects of these mixtures in the following manner: Serial dilutions of potassium ferricyanide and potassium thiocyanate were made in distilled water to give final concentrations of 1:200, 1:400, 1:800, and 1:1600. To sterile test tubes containing 10 cc. of the latter solutions was added 1 cc. of a 1:1000 aqueous solution of a quaternary ammonium solution. The contents of the tubes were mixed thoroughly by swirling and allowed to stand at room temperature for five minutes. One-tenth cubic centimeter of a dilute broth culture of *Staphylococcus aureus No. 209* was added to the mixtures as well as to tubes containing the ferricyanide and thiocyanate dilutions but without the germicide. A control tube of an equal volume of distilled water was also included in the test.

Table I, columns 1 and 2, represents the number of colonies in beef extract agar obtained from one loopful of the test mixtures at time intervals of fifteen and thirty minutes.

Results.—From the data given in Table I we note in no instance any evidence of a neutralizing effect of either potassium ferricyanide or potassium thio-

cyanate upon the germicidal effects of the quaternary ammonium germicide. The amounts of the potassium salts used in the more concentrated solutions far exceeded the quantities theoretically required to combine chemically with molar equivalents of the germicides. Potassium ferricyanide and thiocyanate, in the concentrations used, were in themselves devoid of any antibacterial actions.

Series II.—Several commercially available anionic detergents were tested for their ability to inactivate a quaternary ammonium germicide. The compounds included Duponol C, Santomerse T, and several Nacconols (NR, NRSF, SNO). The results of a typical series of tests in which the first two anionic compounds were studied are given in Table I, columns 3 and 4.

Results.—From the data presented in the table, the anionic detergents give a suggestion of a neutralizing effect upon the antibacterial effects of the quaternary ammonium germicide. It is of interest to note that in many instances, while the anionic-cationic mixtures fail to destroy the test organisms completely, i.e., one neutralizing the other, the anionic preparations when used alone in the control prove to be bactericidal for the test organism. Since, however, there was evidence that under the most suitable conditions existing in any one instance, complete inactivation of antibactericidal activity was not accomplished in any of the anionic-cationic mixtures, additional studies were not made on other groups of synthetic anionic detergents.

Series III.—Salts of many of the high molecular weight naphthalene-sulfonic acids and naphthalene-sulfonates are known to cause a marked precipitate when added to solutions of quaternary ammonium salts. Several of the compounds were tested, therefore, for a possible neutralizing action upon the cationic germicides. The naphthalene derivatives studied included the following (*a*) 1-naphthylamine-4-sulfonic acid; (*b*) 1-naphthylamine-5-sulfonic acid; (*c*) 1-naphthylamine-1,5-disulfonic acid; (*d*) 2-naphthylamine-6,8-disulfonic acid; (*e*) 1-naphthylamine-3,6-disulfonic acid; (*f*) 1-naphthylamine-4,6,8-trisodium sulfonate; (*g*) Na-2-amino-8-hydroxynaphthalene-3,6-disulfonate; (*h*) Na-1-hydroxy - 7 - acetylaminonaphthalene - 3,6 - disulfonate; (*i*) Na-symmetric *bis*(*meta*-amino-benzoyl-*meta* - amino - *para* - methyl - benzoyl - 1-naphthylamino-4,6,8-trisulfonic acid) carbamide.

The results of the effects of solutions of two of the most promising compounds (*g* and *i*) upon the germicide are given in Table I, columns 5 and 6.

Results.—Compound (*g*), in the concentrations used, appeared to have a suggestion of a neutralizing action upon the antibacterial effects of the quaternary ammonium germicide. Attention is again pointed to the fact that, as in the results obtained with two anionic detergents, this naphthalene derivative proved to be antagonistic to the test organism when used in the absence of the quaternary compound. Further studies on naphthalene (*g*), therefore, did not appear to be warranted.

Encouraging results were obtained with the naphthalene derivative, (*i*). As evidenced from the data in column 6, the compound more closely approached the requisites for an ideal quaternary ammonium inactivator than did any of the compounds thus far studied. Naphthalene (*i*) in itself showed no evidence of any adverse action against the test organism even when allowed to act for a period of thirty minutes. This compound was studied more extensively as indicated in a later section of the communication.

Series IV.—Soaps are known to have a marked deleterious effect upon the antibacterial effects of quaternary ammonium solutions. This neutralizing action is due to the combining actions of the high molecular weight fatty acid sodium or potassium salts of soap (anionic detergents) with the quaternary ammonium compounds (cationic detergents). In the present study, the effects of castile and U. S. P. green soap were tested against alkyldimethylbenzylammonium chlorides. Certain modifications were made in the test procedure described previously which included the following: One cubic centimeter of the desired concentration of soap solution was placed in a cotton-plugged test tube. To the soap was added 3 cc. of a saline solution (0.85% NaCl) and the mixture autoclaved at fifteen pounds for fifteen minutes. One cubic centimeter of a 1:5000 dilution of the germicide was added to the cool soap-saline solution and immediately inoculated with 0.1 cc. of a 1:500 dilution of *S. aureus*. The contents of the tubes were mixed thoroughly by shaking, and a 4-mm. loopful plated in agar at the end of one hour and again after four hours. The results of this study are presented in Table II.

TABLE II.—EFFECT OF SOAP UPON ANTIBACTERIAL ACTION OF BENZALKONIUM CHLORIDE (1:25,000) AGAINST *S. aureus*—COLONIES PER 4-MM. LOOP

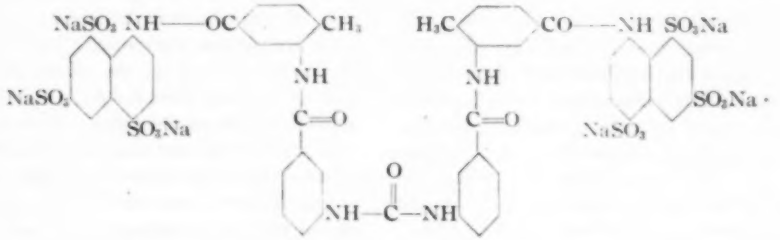
Mg. Soap per 5 Cc.	Inactivator	1:25,000 Quaternary 1 Hr.	1:25,000 Quaternary 4 Hr.	Soap (Only) 1 Hr.	Soap (Only) 4 Hr.
100.00	Castile Soap	20	2	14	0
50.00		22	2	9	0
25.00		41	5	22	0
12.25		39	4	11	1
6.12		82	31	44	9
3.06		83	25	47	6
100.00	U. S. P. Green	3	0	0	0
50.00	Soap	3	0	0	0
25.00		5	0	0	0
12.25		18	0	1	0
6.12		23	0	0	0
3.06		5	0	5	0
Control	No Inactivator and No Quaternary	197 168	97 116		

Results.—From the data given in the table, it is apparent that neither castile nor U. S. P. green soap can be used as neutralizer for the quaternary ammonium germicide. While both soaps show evidence of an action against the germicide, this effect

is not sustained for any length of time and, furthermore, both agents in themselves are bactericidal for the test organism.

METHOD II

With the evidence presented thus far the most promising data obtained in the series of compounds studied were the action of naphthalene (*i*) (Series III) upon the quaternary ammonium germicide. The compound is the sodium salt of symmetric *bis*-(*meta*-amino-benzoyl-*meta*-amino-*para*-methyl-benzoyl-1-naphthylamino-4,6,8-trisulfonic acid) carbamide and has the following structural configuration:



It occurs as a white powder which dissolves readily in cold water. Aqueous solutions are neutral in reaction and remain colorless even after autoclaving. Upon standing at room temperature for a period of several weeks, however, the solutions may assume a pink to deep red color.

The compound was first described by Heymann, Kothe, *et al.* (17) as a specific chemotherapeutic agent for the prophylaxis and treatment of African sleeping sickness caused by trypanosomes. The drug is known in the United States as "Suramin Sodium" (U. S. P. XIII) and in certain foreign countries as "Germanin" or "Bayer 205."

Tiedeman, Fuchs, *et al.* (18), have proposed a method for controlling sanitation of food utensils. In the procedure, use is made of swab containers containing 4 cc. of sterile 0.1% normal sodium thiosulfate solution (chlorine inactivator) buffered at *p*H 7.2. One cubic centimeter of the solution is used for each utensil to be swabbed, i.e., 4 cc. for four utensils. In the present study, Suramin Sodium was evaluated as an inactivator for quaternary ammonium germicides in the presence of the buffer solution in the following manner. To a series of tubes were added 3 cc. of buffer and 1 cc. of a dilution of Suramin Sodium. The tubes were autoclaved at fifteen pounds for fifteen minutes and upon cooling to each was added 1 cc. of a 1:1000 aqueous solution of Benzalkonium Chloride, *p*-tertiaryoctylphenoxy-ethoxyethyldimethylbenzylammonium chloride or cetylpyridinium chloride, and 0.1 cc. of a 1:500 saline dilution of *S. aureus*. The contents of the tubes were mixed thoroughly by swirling and allowed to stand at room temperature. Table III presents the number of colonies in beef extract agar obtained from one loopful of the test mixtures when sampled at the end of one hour and again after four hours.

Results.—It is evident from the data given in Table II that within the limits of error in the sampling of the test solutions Suramin Sodium will definitely inactivate the antibacterial actions of the three quaternary ammonium compounds. Furthermore, no appreciable differences in counts were noted between the one-hour and four-hour test periods on the samples containing the higher concentrations of the inactivator.

METHOD III

In the methods described thus far, the inactivator and quaternary ammonium compounds were mixed just prior to the addition of the test organisms. It appeared desirable, therefore, to determine the neutralizing effects of Suramin Sodium upon a germicide after the latter had been allowed to act

TABLE III.—INACTIVATION OF BENZALKONIUM CHLORIDE (I), *p*-TERTIARYOCTYLPHENOXY-ETHOXYETHYL-DIMETHYLBENZYLAMMONIUM CHLORIDE (II), AND CETYL PYRIDINIUM CHLORIDE (III) AGAINST *S. aureus*—COLONIES PER 4-MM. LOOP

Mg. Suramin Sodium per 5 Cc.	1:5000 Quaternary Ammonium Compound					
	I		II		III	
	1 Hr.	4 Hr.	1 Hr.	4 Hr.	1 Hr.	4 Hr.
50	280	258	263	219	151	158
40	213	319	290	165	77	54
30	248	252	209	180	85	54
20	230	263	237	93	50	48
10	190	203	231	55	47	7
5	166	94	162	71	16	3
2.5	100	32	252	28	24	0
1	175	166	171	58	126	79
Control—No Inactivator No Quaternary	308	320	293	274	372	344

TABLE IV—ACTION OF SURAMIN SODIUM UPON BENZALKONIUM CHLORIDE AFTER LATTER HAS BEEN ACTING UPON *S. aureus*—COLONIES PER 4-MM. LOOP

Quaternary Dilution per 1 Cc.	Inactivator—0.05 Gm. per 4 Cc. —Culture Dilution— 1:500[a]	Undiluted	No Inactivator —Culture Dilution— 1:500	Undiluted
1:1,000	0	0	0	0
1:5,000	0	0	0	0
1:10,000	0	190	0	9
1:20,000	0	630	0	45
1:30,000	0	704	0	524
1:40,000	0	+++	0	++
1:50,000	3	++++	0	+++
1:60,000	5	++++	0	++++
1:70,000	4	++++	0	++++
1:80,000	46	++++	25	++++
1:90,000	66	++++	5	++++
1:100,000	68	++++	24	++++
None	300	++++	130	++++

[a] 1:500 = 0.1 cc. of 1:500 culture of *S. aureus* to 1 cc. Benzalkonium chloride.
Undiluted = 0.1 cc. of undiluted culture of *S. aureus* to 1 cc. Benzalkonium chloride.
++ to ++++ = Too many to count, yet numbers may be less (++, +++) than those noted in many of the other plates showing maximum number of colonies (++++).

upon the bacteria. This was carried out as follows: To 1-cc. quantities of a series of dilutions of Benzalkonium Chloride was added 0.1 cc. of a 1:500 saline dilution of *S. aureus*. A second series received 0.1 cc. of an undiluted broth culture of the organisms. The mixtures were allowed to stand for thirty minutes at which time 4 cc. of a 1:80[1] dilution of Suramin Sodium was added to each tube. The contents of the tubes were mixed thoroughly and again allowed to stand for thirty minutes. A 4-mm. loopful of the test suspensions was then plated in agar and incubated as before. The results of this study are presented in Table IV.

Results.—In summarizing Table IV the following may be noted: In the absence of Suramin Sodium the limiting dilution of the quaternary ammonium germicide that apparently destroyed the dilute suspension (1:500) of test organisms was 1:70,000. In the presence of the inactivator, however, complete absence of growth was first noted in the test medium containing 1:40,000 of the cationic detergents. Yet dilutions in the latter series up to and including 1:70,000 showed evidence of only a few organisms in the test sample. This again emphasizes the high bactericidal activity of quaternary ammonium germicides.

In the series inoculated with 0.1 cc. of undiluted culture of *S. aureus*, complete absence of growth was noted in the presence or absence of the inactivator in the 1:5000 dilution of Benzalkonium Chloride. The neutralizing action of the inactivator upon the germicide is again evidenced by the greater number of colonies that developed in its presence as compared to the relatively few that grew out in the 1:10,000 and 1:20,000 samples of the germicides in the absence of neutralizer.

[1] 1:80 = 0.0125 Gm./cc., or 0.050 Gm./4 cc. of solution.

In studies entirely independent from these, Gilcreas (19) found that Suramin Sodium would effectively neutralize a quaternary ammonium germicide in the presence of *Escherichia coli* and *Staphylococcus albus* as test organisms. Furthermore, this inactivation was sustained for a period of twenty-four hours. Favorable results on the use of the inactivator have also been obtained by Jamieson (20), Weber and Black (21), and Mueller, Seeley, and Larkin (22).

SUMMARY

A résumé has been presented on the necessity for providing a suitable means for inactivating the high antibacterial activity of quaternary ammonium germicides.

Synthetic anionic detergents, as well as soaps, fail to completely inactivate the surface-active cationic detergents.[2]

A group of sulfonates and sulfonic acids were studied for their possible application as inactivators for the germicides. Suramin Sodium, a sulfonic acid derivative, more closely approached the requirements for an inactivator for quaternary ammonium compounds.

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[2] Quisno, Gibby, and Foter (23) in a recent publication appearing after the present study had been completed, described a medium containing lecithin and "Tween 80" (a polyoxyethylene derivative of sorbitan monooleate) for the inactivation of relatively high concentrations of quaternary ammonium salts. When used alone the medium was also found to promote the growth of some strains of bacteria.

New Syntheses of Caffeine and Theophylline*

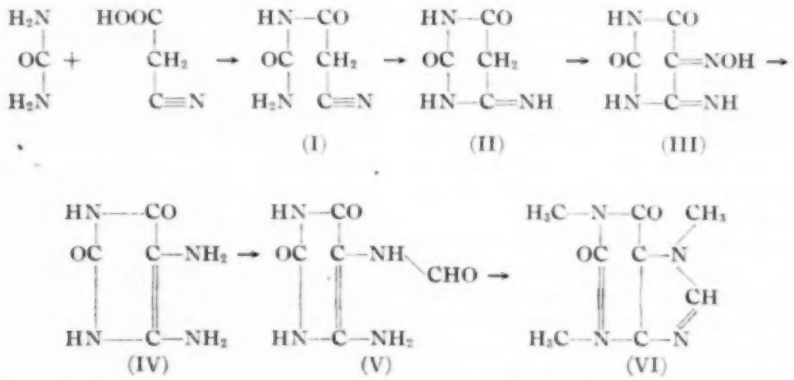
By BOGUSLAW BOBRANSKI and ZDZISLAW SYNOWIEDSKI†

Starting with urea and cyanacetic acid, caffeine and theophylline have been prepared by convenient modifications of methods previously described for these syntheses.

AFTER its structure was established, a synthesis of caffeine was described by E. Fischer (1). This synthesis, from dimethylurea and malonic acid dichloride, was important only for its theoretical value. For practical use, it is preferable to start with uric acid which is now produced from guano.[1]

As a result of war conditions in 1942 there was an extreme shortage of caffeine in Poland. In order to provide caffeine it was necessary to develop a convenient and economical synthesis of this alkaloid. In connection with this problem the Traube (2) synthesis of caffeine from mono- or dimethylurea was considered. To avoid the inconvenience of producing these substances, urea was used as the starting product and the methyl groups were introduced in the further stages of the synthesis. Cyanacetic urea (I) was produced by condensation of urea with cyanacetic acid in the presence of acetic anhydride (3) which was immediately transformed by action of 40 per cent sodium hydroxide into 4-imino-2,6-dioxyhexahydropyrimidine (II). The latter, without being isolated, was converted by action of nitrous acid into iminovioluric acid (III).

The iminovioluric acid (III) was reduced by means of zinc powder and sulfuric acid (4) to the sulfate of the diamine (IV). This base was converted by heating with anhydrous sodium formate and formic acid into the 4-amino-5-formylamino-2,6-dioxypyrimidine (V), which was isolated. The direct methylation of (V) with 2 moles of dimethylsulfate did not yield the expected 1,3-dimethyl derivative, but a mixture of dimethyl derivatives. Upon heating the solution containing this mixture with sodium hydroxide and then adding an additional mole of dimethyl sulfate, caffeine (VI) was formed.



Thus (V) in a simple procedure was converted directly into caffeine (VI). The complete synthesis of caffeine consists consequently of four operations: (*a*) the preparation of iminovioluric acid (III); (*b*) the preparation of 4,5-diamino-2,6-dioxypyrimidine sulfate (IV); (*c*) the formylation of the diamine; (*d*) the preparation of the caffeine from the formylamine.

Efforts to synthesize theophylline by a method analogous to that used for caffeine were unsuccessful. Neither could 4-imino-5-formylamino-2,6-dioxypyrimidine (V) be converted into the 1,3-dimethyl derivative

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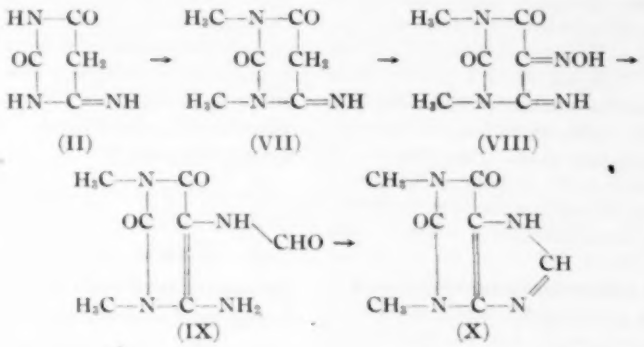
[1] For a review of methods of synthesis of caffeine see H. Schwaneberg, *Pharm. Ind.*, **10**, 109–11(1943).

with a satisfactory yield. The sodium salt of 4-imino-2,6-dioxy-hexahydropyrimidine (II), however, was successfully methylated with dimethyl sulfate at 0°, a reaction not previously accomplished, as is mentioned by Max Conrad (5). The 1,3-dimethyl-4-imino-2,6-dioxyhexahydropyrimidine (VII) obtained by this reaction was converted into 1,3-dimethyliminovioluric acid (VIII) and the latter by treatment with zinc and formic acid transformed directly into the 1,3-dimethyl - 4 - amino - 5 - formylamino - 2,6-dioxytetrahydropyrimidine (IX) which is converted into theophylline (X) by heating in a sodium hydroxide solution (6). Thus the synthesis of theophylline was realized in 5 operations:



EXPERIMENTAL

Preparation of Iminovioluric Acid.—A mixture of 950 Gm. of chloracetic acid and 300 cc. of water was neutralized with a 40% solution of sodium hydroxide (about 750 cc.) using phenolphthalein as the indicator. A solution of 510 Gm. of sodium cyanide in 1 L. water at a temperature of 50° was added to this mixture, and the temperature was kept below 80° during the reaction. The mixture, at the end of the reaction, was cooled to 30° and acidified with 30% sulfuric acid (about 1.55 Kg.) using Congo red as indicator. To the cold cyanacetic acid solution was added 600 Gm. of urea and the resulting solution (about 5 L.) evaporated under diminished pressure to dryness. The residue was cooled, 1500 cc. of acetic anhydride was added, and the mixture was stirred from time to time and cooled so that the temperature did not rise above 70°. The reaction product was allowed to stand overnight, after which the acetic acid formed was removed completely by distillation under diminished pressure. To the dry residue 2.3 L. of a 40% solution of sodium hydroxide was added with stirring, the temperature being maintained below 60° during the reaction. After two to three hours the sodium salt of 4-imino-2-6-dioxyhexahydropyrimidine separated from the reaction mixture. Then 725 Gm. of sodium nitrite dissolved in 3 L. water was added, and into this mixture a 10% sulfuric acid solution was introduced to obtain a faintly acid reaction with litmus and neutral reaction with Congo red. A red microcrystalline iminovioluric acid was obtained which, after standing for twelve hours, was removed by filtration and washed with 1500 cc. of water. Yield: 1 Kg.; 64%.

Preparation of 4,5-diamino-2,3-dioxypyrimidine sulfate.—One kilogram of iminovioluric acid was suspended in 20 L. of 20% sulfuric acid. The mixture was stirred vigorously and 1 Kg. of zinc powder was introduced in small portions with cooling so that the temperature did not rise higher than 30°. The stirring was continued during one hour, the mixture cooled, and the crude 4,5-diamino-2,3-dioxypyrimidine sulfate filtered off, washed with 0.5 L. water, and dried at 40°. Yield: 1 Kg.; 76%.

Preparation of 4-Amino-5-formylamino-2,6-dioxypyrimidine.—One kilogram of dry, crude 4,5-diamino-2,6-dioxypyrimidine sulfate was mixed thoroughly with 0.6 Kg. of dry sodium formate, 5 L. of formic acid was added, and the mixture was heated during four to five hours on a steam bath under a reflux condenser. After standing for twelve hours at room temperature the crude formyl derivative was removed by filtration, washed several times with water and finally dried. Yield: 750 Gm.; 90.5%.

Preparation of Caffeine.—Seven hundred fifty grams of finely powdered, crude 4-amino-5-formylamino-2,6-dioxypyrimidine was introduced into a round-bottomed flask containing 2.95 L. of 3 *N* sodium hydroxide solution, 1.12 Kg. of dimethyl sulfate was added with stirring, the temperature being maintained below 40°. After all the dimethyl sulfate was added the stirring was continued for one and one-half hours and then 1.39 L. of 3 *N* sodium hydroxide solution was added. The reaction mixture was heated rapidly to 90° and the stirring continued another half hour. After cooling to room temperature, 0.57 Kg. of dimethyl sulfate was added slowly with stirring. After one hour the mixture was filtered and the filtrate was concentrated

under diminished pressure to 3 L. Upon standing for forty-eight hours the crude caffeine (about 300 Gm.) crystallized and was removed by filtration. From the filtrate, by extracting 5 times with 200 cc. of chloroform, an additional 150 Gm. of crude caffeine was obtained. The crude caffeine was recrystallized 4 times from water, the hot solutions being decolorized with charcoal. Yield: 350 Gm.; 37.5%. The over-all yield of caffeine was 16.5% of theory based on the starting product.

Preparation of 4-Imino-2,6-dioxyhexahydropyrimidine.—This product was obtained by the procedure outlined for the preparation of iminovioluric acid. The sodium salt of 4-imino-2,6-dioxyhexahydropyrimidine which is formed intermediately by condensation of cyanacetylurea by means of sodium hydroxide at 60° was dissolved in 8 L. of water and the crude 4-imino-2,6-dioxyhexahydropyrimidine was precipitated by acidification of the solution with acetic acid. Yield: 710 Gm.; 56%.

Preparation of 1,3-Dimethyl-4-imino-2,6-dioxyhexahydropyrimidine.—Seven hundred ten grams of 4-imino-2,6-dioxyhexahydropyrimidine was suspended in 3.73 L. of 3 *N* sodium hydroxide solution. After cooling to 0°, 1.58 Kg. (1.19 L.) of dimethyl sulfate was added and the mixture stirred vigorously for five hours at 0°. The crystalline 1,3-dimethyl derivative formed was filtered off. From the filtrate upon acidification with acetic acid the unchanged 4-imino-2,6-dioxyhexahydropyrimidine was recovered and methylated as described above. Yield: 530 Gm.; 61%.

Preparation of 1,3-Dimethyliminovioluric acid.—Five hundred thirty grams of 1,3-dimethyl-4-imino-2,6-dioxyhexahydropyrimidine was dissolved in 16 L. of boiling water, 260 Gm. of sodium nitrite was added, and the hot solution was treated with 270 Gm. of 90% acetic acid. Upon cooling, the purple-colored crystalline 1,3-dimethyl-4-iminovioluric acid was collected on a filter and washed with about 1 L. of water. Yield: 530 Gm.; 84.5%.

Preparation of 1,3-Dimethyl-4-amino-5-formylamino-2,6-dioxytetrahydropyrimidine.—Five hundred thirty grams of 1,3-dimethyl-4-iminovioluric acid was dissolved in 5.3 Kg. of 95% formic acid and to this solution, while being stirred rapidly, 430 Gm. of zinc powder in small portions was added. The reaction mixture was cooled to 30°. As soon as the red color of the solution had disappeared (after two to three hours) the reaction mixture was refluxed for three hours. Upon cooling the zinc formate separated, was filtered off, and washed with 0.5 Kg. of 95% formic acid. From the filtrate the formic acid was removed by distillation under diminished pressure. The residue was dissolved in 8 L. of water at 80–90° and filtered from the insoluble zinc formate. A yield of 265 Gm. of 1,3-dimethyl-4-amino-5-formylamino-2,6-dioxyhexahydropyrimidine crystallized from the filtrate. The mother liquor upon concentration to 1 L. yielded an additional 120 Gm. of the same product. Yield: 385 Gm.; 62.5%.

Preparation of Theophylline.—Three hundred eighty-five grams of 1,3-dimethyl-4-amino-5-formylamino-2,6-dioxytetrahydropyrimidine was suspended in 3.85 L. of hot water and 310 cc. of a 40% solution sodium hydroxide was added. The solution was heated on a water bath to 90° for ten minutes and upon cooling 230 cc. of concentrated hydrochloric acid was added. The still alkaline solution was treated with 30% acetic acid to weakly acid reaction (about 14 cc.). The theophylline crystallized after some hours, was filtered off, and washed with 300 cc. of water. The crude product (300 Gm.) was dissolved in 6 L. of boiling water and the solution decolorized with charcoal and allowed to stand for several hours for crystallization. Two hundred eighty grams of pure theophylline was obtained (80% theoretical). The over-all yield of theophylline was 14.3%.

SUMMARY

1. A new method for the synthetic preparation of caffeine from urea and chloracetic acid is described.

2. The conversion of 4-amino-5-formylamino-2,6-dioxypyrimidine into caffeine is described.

3. The methylation of 4-amino-2,6-dioxyhexahydropyrimidine in position 1 and 3 by means of dimethyl sulfate is described. Thus a simple method for the preparation of theophylline from urea and chloracetic acid as starting materials has been developed.

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The Fractionation by Alcohol of Equine Antitoxic Plasma*

By ALFRED H. FOWELL and FREDERICK F. JOHNSON

The desirability of concentrating equine antitoxin relatively free from pyrogen prompted an investigation of the use of alcohol fractionation at reduced temperatures. In normal antitoxic plasma the antitoxin is found in the T-globulin and gamma-globulin components. In pepsin digested antitoxic plasma the antitoxin is found in a modified component formed by the merger of the "T" and "gamma." By the alcohol fractionation methods described, either the "T + gamma" or the new modified component can be obtained in a satisfactory state of purity as shown by electrophoretic analyses. The methods have been applied to the preparation of diphtheria and vibrion septique antitoxin.

VARIOUS workers (1–4) have reported that normal horse plasma can be separated electrophoretically into the following six well-characterized plasma proteins: albumin, α_1-globulin, α_2-globulin, β-globulin, fibrinogen, γ-globulin. The concept that most antibodies are found associated with the γ-globulin fraction has been repeatedly verified in recent years. Van der Scheer and co-workers (5, 6) reported that hyperimmunized horse plasma contains an additional electrophoretic component which they termed T-globulin and which exhibited an electrophoretic mobility between that of the β-globulin and γ-globulin. They concluded that although the T-globulin "peak" became much larger when a large amount of antitoxin appeared in the serum, the area of the peak could not be taken as proportional to the measured antitoxin activity. Nevertheless, the T-globulin undoubtedly contains the major portion of the antitoxin of a hyperimmune serum or plasma.

The usual processes for preparing antitoxin for clinical administration employ a partial enzyme digestion of the horse protein. This digestion has been conducted at temperatures ranging from 0° to 60° (7–9). Under most conditions, the antitoxin is more stable to enzyme action than is the inert protein; thus an enzyme digestion step, when used in conjunction with the usual procedures for purifying antitoxin by salt fractionation, generally results in a greatly increased purification. Coghill, *et al.* (10), reported that enzyme (Takadiastase) digestion so alters the antitoxin that its horse protein specificity is greatly reduced.

Van der Scheer and Wyckoff (11, 12) followed the course of peptic digestion of tetanus antitoxic horse serum with electrophoretic analyses and showed that during digestion the T-globulin is rapidly converted into a slower moving component of mobility similar to that of γ-globulin; in other words, the T- and γ-globulins are said to merge. Deutsch, *et al.* (13), working with human γ-globulin, have reported conditions for peptic digestion which result in splitting the γ-globulin molecules into half molecules without destroying the antibody activity.

The fractionation of human plasma proteins by employing isoelectric precipitation with varying conditions of ethanol concentration and ionic strength at reduced temperatures has been described by Cohn (14). The conditions for separation and purification of human γ-globulin have been further developed by Deutsch (15) who employed ionic strengths as low as 0.01. Smith and Gerlough (16) applied the Cohn (14) fractionation scheme to hyperimmune tetanus horse plasma and found the antitoxic activity to be distributed between at least three distinct proteins: T-globulin, γ-globulin, and a β-globulin.

We have attempted to devise a set of conditions where, by varying the *p*H, ionic strength, temperature, and ethanol content of horse plasma, the antitoxic globulins can be removed in a state of suitable purity. Cold alcohol fractionation methods are

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easily applicable to large-scale preparation, and they can be applied with less risk of pyrogen formation than the conventional salt fractionation procedures. We have applied alcohol fractionation to both pepsin digested and undigested horse plasma and have studied the pepsin digestion of antitoxic fractions subsequent to their separation. Whether or not the enzyme digestion of an equine antitoxin actually results in a lower incidence of "serum sickness" is in our opinion still open to question, since the reported clinical comparisons generally have been performed with undigested antitoxin of average potency and digested antitoxin of higher potency. Since the alcohol fractionation methods can yield antitoxin of potency equivalent to that obtained from salt enzyme procedures, it should be possible to compare clinically the effects of digested and undigested antitoxin of equal horse protein content per dose.

EXPERIMENTAL

Methods.—The antitoxic plasmas were prepared by usual methods, were of low to average potencies, and were taken from pools each representing five or more horses bled in a citrate anticoagulant. Diphtheria antitoxin assays were made by the Ramon flocculation technique (17) except where otherwise stated.

The fractionation conditions of *p*H and ionic strength were measured or calculated, respectively, on the basis of the volume obtained after the addition of alcohol. The *p*H of alcoholic solutions was determined on undiluted aliquots at 10°. Alcohol concentrations are expressed as per cent by volume. When adding alcohol to an aqueous protein solution or one of low alcohol content, the heat of dilution was minimized by previously diluting the alcohol to 50% and cooling to below —15°. All alcohol additions were gradual with vigorous stirring.

Electrophoretic analyses were made with the standard Tiselius apparatus using the Longsworth scanning device. The runs were made at 0.7° using a diethylbarbiturate buffer of *p*H 8.6 and ionic strength 0.10.

All protein precipitates were analyzed after being dried from the frozen state and being dissolved in 0.9% sodium chloride between *p*H 7.0–7.4.

Fractionation of Heat-Defibrinated Plasma.—Antidiphtheria horse plasma was held at 56° for fifteen minutes. The precipitate was removed by filtration and the filtrate was divided into four 45-cc. aliquots each of which was diluted with water to a volume of 100 cc. Four successive precipitates were formed from each aliquot under the conditions given in Table I. For example, from aliquot I, precipitate IA was formed and removed, then precipitate IB was formed from the effluent and removed, etc. The plasma used had a potency of 650 Lf/cc. The best precipitate in this group was IIIB which had a potency of 14,240 Lf/Gm. and contained 67% cent of the original unitage.

TABLE I.—FRACTIONATION OF HEAT DEFIBRINATED PLASMA

Plasma	*p*H	EtOH, %	Temp., °C	Dry Wt., Gm.	Units	Units/Gm.
IA	6.28	16	0	0.7956	2,387	3,000
IB	6.28	19	−2	1.124	15,400	13,700
IC	6.25	22	−3	0.2698	3,632	13,460
ID	6.28	25	−5	0.1265	1,054	8,333
IIA	6.40	16	0	0.6849	1,750	2,500
IIB	6.53	19	−2	1.199	15,490	12,910
IIC	6.54	22	−3	0.4193	7,425	17,700
IID	6.52	25	−5	0.0961	837	8,721
IIIA	6.79	16	0	0.6727	2,018	3,000
IIIB	6.85	19	−2	1.378	19,610	14,240
IIIC	6.81	22	−3	0.1330	2,163	16,260
IIID	6.82	25	−5	0.0715	392	9,677
IVA	7.48	16	0	0.2530	781	3,086
IVB	7.48	19	−2	1.333	15,620	11,790
IVC	7.55	22	−3	0.2586	2,068	8,000
IVD	7.52	25	−5	0.1294	1,078	8,333

Fractionation of Unmodified Plasma.—Antidiphtheria horse plasma, without defibrination or enzyme digestion, was fractionated by manipulation of the four variables (ethanol concentration, *p*H, ionic strength, temperature) listed in Table II. Fractions 1 through 3 were obtained as successive precipitates from one aliquot; fractions 4 through 7 were obtained as successive precipitates from another aliquot. Fractions 8 through 11 were obtained from four aliquots of the effluent from fraction 7, and in each case consisted of essentially all T-globulin and γ-globulin. The units in Table II were obtained by the standard guinea-pig protection test.

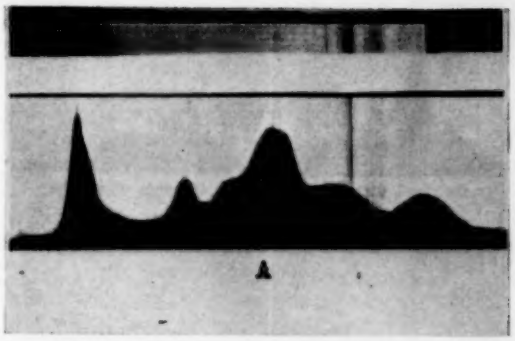
A study of the results shown in Table II indicated that the best approach, within the range of conditions studied, would be to discard a precipitate

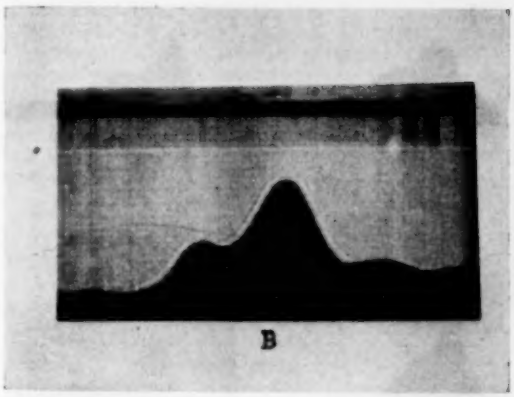
formed under the conditions of fraction 6, and then to precipitate the antitoxin under the conditions of fraction 11. Further experimentation involving minor variations of these conditions led to the construction of what we term Method 1, the method being applicable to the fractionation of whole undigested antitoxic horse plasma. Electrophoretic patterns of the products obtained by Method 1 show them to consist of T-globulin plus smaller amounts of γ-globulin and β-globulin. A typical electrophoretic pattern is shown in Fig. 1. By Method 1, an antidiphtheria plasma of low potency (ca. 400–500 u./cc.) yields about 65% of the antitoxin of a potency of about 15,000 u./Gm. An antidiphtheria plasma of average potency (ca. 600–700 u./cc.) yields about 80% of the antitoxin of a potency of about 20,000 u./Gm.

Statement of Method 1.—Dilute each liter of plasma with about 1900 cc. of 1° water. By the use of cold 50% ethanol, *p*H 4 acetate buffer, and sodium chloride, adjust to the following conditions: ethanol 12%, *p*H 5.10, ionic strength 0.057, temperature −2°. Remove the precipitate by centrifugation at −2° and discard. Filter the effluent and adjust to *p*H 7.4 with 2 *N* NaOH. By the use of cold 95% ethanol, adjust to the following conditions: ethanol 34%, *p*H 7.40, ionic strength 0.059, temperature −5°. Remove the precipitate by centrifugation at −5°, suspend in a minimum quantity of water, and dry by vacuum from the frozen state.

Experiments on Enzyme Digestion.—A 2% solution of antitoxin (19,200 u./Gm.) prepared by Method 1 was subjected to pepsin digestion at low temperatures and low *p*H. Variations of time, amount of pepsin, temperature, and *p*H between 2.0 and 4.0 were tried, but in all cases a loss of activity approximating 50% resulted.

A comparison was made of the pepsin digestion of the same 2% solution of antitoxin and the pepsin digestion of whole plasma diluted to 2% protein, the original citrated horse plasma being 7% protein. The conditions were: temperature 2–5°, *p*H 2.4, 1: 10,000 pepsin 0.2%. Samples were removed at intervals for unitage assay by the Ramon flocculation test. The potency of the 2% antitoxin prepared by Method 1 decreased approximately 50% in thirty





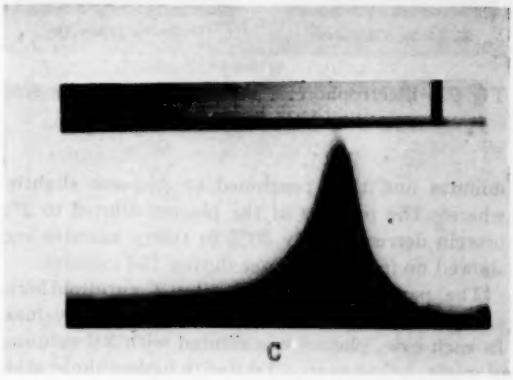


Fig. 1.—Electrophoretic patterns of (A) diphtheria horse plasma, (B) diphtheria antitoxin made by method 1, and (C) diphtheria antitoxin made by method 3.

TABLE II.—FRACTIONATION OF UNMODIFIED PLASMA

	Fraction						Electrophoretic Analysis, %					
	Ethanol Conc., %	*p*H	Γ/2	Temp., °C.	% Yield of Units	Units/Gm.	Alb.	α-Glob.	β-Glob.	Fib.	T	γ-Glob.
1	14	5.1	0.04	−3	55	10,200	6.1	8.1	7.0	[a]	42.8	36.0
2	25	7.2	0.03	−5	7.6	23,300	4.4	12.2	0	0	73.6	9.8
3	40	4.8	0.02	−5	23	4,300	56.8	27.8	0	0	15.3	0
4	8	5.1	0.05	−2	0	0	0	3.9	19.9	76.1	0	0
5	10	5.1	0.05	−3	5.4	4,320	8.7	6.3	0	0	33.6	51.3
6	12	5.1	0.05	−3	11	7,060	...	...	...	...	...	...
7	14	5.1	0.05	−3	26	18,600	8.3	15.0	0	0	54.6	22.1
8	34.8	6.8	0.05	−5	67	17,300						
9	34.6	7.0	0.05	−5	74	17,800						
10	34.6	7.2	0.05	−5	65	17,700						
11	33.8	7.4	0.05	−5	55	21,500						

[a] The fibrinogen in fraction 1 was masked by the T-globulin; the concentration of fibrinogen is therefore included in the concentration ratio for T.

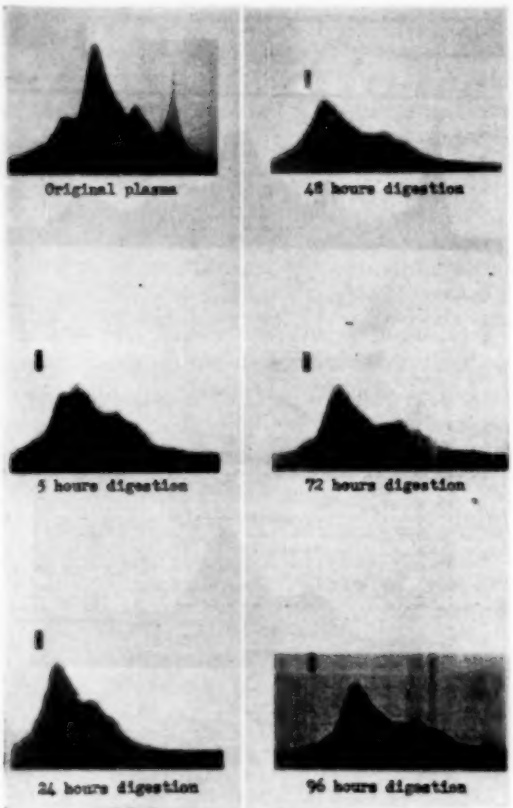


Fig. 2.—Electrophoretic patterns of pepsin-digested diphtheria horse plasma.

minutes and then continued to decrease slightly, whereas the potency of the plasma diluted to 2% protein decreased only 30% in thirty minutes and showed no further decrease during 150 minutes.

The pepsin digestion of diluted antidiphtheria plasma was then studied at three higher *p*H values. In each case, plasma was diluted with 2.6 volumes of water, adjusted to *p*H 6.0 with hydrochloric acid, 0.36 Gm. 1:10,000 pepsin added per 100 cc. of plasma adjusted to the indicated *p*H with hydrochloric acid, and maintained at 2° during digestion. The results in terms of potency and flocculation time are shown in Table III. The results of these digestion studies clearly indicate that within the *p*H range of 3.5 to 4.0 and at a temperature of 2°, the flocculating activity remains constant after one hour, but that a considerable period is required for the flocculating time to drop to a proper value. A low Kf is taken as an indication of sufficient digestion, resulting in the merger of the T and gamma components and presumably the splitting of the antitoxin into half molecules. The merger of these two antitoxin bearing components as measured against time of digestion is exemplified in Fig. 2 which shows the electrophoretic patterns of plasma (diluted with one volume of water) and digested at *p*H 4.0, temperature 2°, for various time intervals. This digestion series was also followed with measurements of the Lf units per cc. and the corresponding Kf values and the results roughly verified those reported in Table III for *p*H 4.00.

Some of the products of the enzyme digestion studies just described were submitted to an arbitrary cold alcohol fractionation, namely, discarding the *p*H 5.1, 15% ethanol, —2° precipitate, and saving the subsequent *p*H 7.4, 35% ethanol, —5° precipitate. Although none of the final fractions were of adequate potency and yield, it was noted that the potency markedly increased with increasing dilution of the plasma before digestion. A plasma originally diluted to about 1 to 7 appeared to allow the most satisfactory fractionation after digestion. On the other hand, a plasma diluted 1 to 7 would never show a merger of the T and gamma components or a low Kf value after digestion for as long as five days at 2° and *p*H 4.0. Apparently the lowering of the ionic strength by diluting the plasma 1 to 7 greatly increased the sharpness of fractionation but prevented an adequate degree of digestion. For this reason, subsequent pepsin digestions were made on plasma diluted 1 to 2, with the dilution being increased after digestion to 7 times the plasma volume.

Fractionation of Pepsin Digested Plasma.—Using diluted antidiphtheria plasma, digested as described above, a large number of fractionations were performed to find suitable conditions for the precipitation of an inert fraction 1. (Conditions for precipitation of the fraction 2 antitoxin could be obtained rather closely from previous data.) The pertinent data for the discard fraction is shown in Table IV. In this case, antidiphtheria plasma was diluted 1 to 2 with water, adjusted to *p*H 6.0, treated with 0.36 Gm. 1:10,000 pepsin per 100 cc. plasma, adjusted to *p*H 4.0 and maintained at 2° for ninety-six hours. The mixture was clarified by continuous Sharples centrifugation (15,000 r.p.m.) at 2° and the effluent was diluted to aproximately seven times the plasma

TABLE III.—PEPSIN DIGESTION OF DILUTED PLASMA

*p*H	Digestion, Hr.	Lf Units/Cc.	Kf, Min.
3.50	0	136	22
	1	104	10
	2	104	11
	3	104	8
	4	104	8
	5	104	6
3.75	0	124	26
	0.5	104	18
	16	104	14
4:00	0	149	20
	0.5	124	18
	1	124	18
	2	124	17
	3	124	17
	4	124	17
	24	124	14
	48	124	10
	72	124	7
	96	124	7

volume. A series of aliquots were adjusted to the conditions of *p*H and ethanol concentration shown in Table IV, the temperature being kept constant at —2° and the ionic strength at 0.015.

From Table IV, the *p*H 4.6, ethanol 20%, —2°, ionic strength 0.015 fraction was selected as a discard fraction affording a satisfactory compromise between loss of inert material and loss of unitage. Subsequent precipitation from the effluent of the fraction 1 at *p*H 7.0, 30% ethanol, and —5° yielded satisfactory antitoxin. The method was tentatively designated as Method 2 and was used in a series of production runs on pyrogenic antidiphtheria and anti-vibrion-septique plasma. In all such runs, antitoxin of a low pyrogenicity was obtained in yields averaging 53% of the starting unitage.

The chief disadvantage of Method 2 is the large volumes which must be handled since the low ionic strength is attained by dilution. This disadvantage can be overcome by precipitating a total globulin fraction and redissolving it in salt-free water to an appropriate volume.

The conditions for precipitating a total globulin fraction were taken from the data in Table V. Antidiphtheria plasma was pepsin digested in the usual manner and aliquots were brought to the fractionation conditions of Table V with a constant temperature of —5° and a constant ionic strength of 0.07. The per cent yields of activity are based on the activity of the original plasma which loses approximately 30% of its activity during pepsin digestion.

The conditions selected for the total globulin precipitation were: *p*H 6.6, ethanol 35%, temperature —5°, ionic strength 0.07. A large total globulin fraction was prepared by these conditions and was redissolved in sufficient 2° water to bring the ionic strength to approximately 0.027. By adjusting the *p*H to 5.1 at +2°, a large amount of low potency material (2500 Lf u./Gm.) separated and was discarded by centrifugation. This first discard fraction was designated fraction 1*a*. Equal aliquots of the effluent from fraction 1*a* were then adjusted to the fractionation conditions described in Table VI at a constant ionic strength of 0.017.

TABLE IV.—PRECIPITATION OF DISCARD FRACTION FROM PEPSIN DIGESTED ANTIDIPHTHERIA PLASMA AT —2° AND IONIC STRENGTH 0.015

Fraction			
*p*H	EtOH, %	Yield, Gm.	Lf Units/Gm
4.2	20	0.3301	<1182
4.2	25	0.3708	<1610
4.2	35	0.3708	<1610
4.6	10	0.1499	<1240
4.6	15	0.2796	<1331
4.6	20	0.4279	1766
4.8	10	0.1940	< 960
4.8	15	0.3684	1234
4.8	20	0.5438	2933
5.0	10	0.2410	<1079
5.0	15	0.4225	2359
5.0	20	0.5809	4625

TABLE V.—PRECIPITATION OF TOTAL GLOBULIN FRACTION FROM PEPSIN DIGESTED ANTIDIPHTHERIA PLASMA AT —5° AND IONIC STRENGTH 0.07

Fraction		% Yield	
*p*H	EtOH, %	of Units	Lf Units/Gm.
6.6	30	65.6	7194
7.0	30	62.1	7291
7.2	30	62.8	7316
6.6	35	68.3	7071
6.8	35	62.4	7088
7.0	35	62.9	7203
7.2	35	59.5	6930

TABLE VI.—PRECIPITATION OF SECOND DISCARD FRACTION (1*b*) FROM PEPSIN DIGESTED ANTIDIPHTHERIA PLASMA AT IONIC STRENGTH 0.017

Fraction				
*p*H	EtOH, %	Temp., °C.	Yield, Gm.	Lf Units/Gm
4.4	5	—1	0.4200	3500
4.6	5	—1	1.006	5404
4.8	5	—1	1.474	6194
4.4	10	—1	0.9501	4010
4.6	10	—1	1.425	6090
4.8	10	—1	2.053	6679
4.4	15	—2	1.639	4453
4.4	20	—2	1.925	5518
4.6	15	—2	2.302	4728
4.6	20	—2	2.917	6615

TABLE VII.—PRECIPITATION OF ANTITOXIN (FRACTION 2) FROM EFFLUENT 1*b* AT —5° AND IONIC STRENGTH ABOUT 0.02

Fraction			
*p*H	EtOH, %	Yield, Gm.	Lf Units/Gm.
6.7	20	2.231	16,800
6.7	30	2.279	16,811
6.7	40	2.314	...
7.0	20	1.608	17,150
7.0	30	2.170	17,500
7.0	40	2.258	17,400
7.2	20	1.760	18,618
7.2	30	2.077	18,641
7.2	40	2.172	18,666
7.4	20	1.682	18,323
7.4	30	2.194	19,059
7.4	40	2.284	18,865

The conditions selected from Table VI for the precipitation of discard fraction 1*b* were: *p*H 4.4, ethanol 20%, temperature —2°, ionic strength 0.017. A large portion of plasma was processed to the effluent 1*b* stage and various equal aliquots were used to determine the proper conditions for precipitation of fraction 2 (digested antitoxin). The conditions of *p*H and alcohol concentration are given in Table VII. The temperature was maintained constant at —5°, and the ionic strength being relatively immaterial at this stage was uncontrolled but was within the region of 0.02. From the data in Tables V through VII, Method 3 was constructed which yields an enzyme digested alcohol fractionated antitoxin.

Statement of Method 3.—Add an equal volume of 2° water to antitoxic horse plasma. Adjust to about *p*H 6.0 with hydrochloric acid, add 0.36 Gm. 1:10,000 pepsin per 100 cc. of plasma, then adjust the *p*H to 4.0 and maintain at 2° for ninety-six hours. Precipitate the total globulin fraction at 35% ethanol, *p*H 6.6, —5°, and ionic strength 0.073. Collect the total globulin fraction by centrifugation at —5° and redissolve in 2° water, of *p*H 4.0, to the original plasma volume. Adjust the *p*H to 5.1, remove and discard the insoluble material by centrifugation at +2°. Adjust the effluent to *p*H 4.4, ethanol 20%, —2°, ionic strength 0.017. Remove and discard the insoluble material by centrifugation at —2°. Adjust the effluent to *p*H 7.4, ethanol 40% —5°, ionic strength about 0.02. Remove the antitoxic globulin by centrifugation at —5°. Suspend the precipitate in a minimal amount of ice water, freeze, and dry by vacuum from the frozen state.

In the case of antidiphtheria plasma of average potency, about 50% of the activity can be recovered with potency of approximately 20,000 Lf u./Gm. The dried material can be dissolved to the desired concentration in physiological saline or 0.3 molar glycine and can be sterilized by Seitz filtration.

DISCUSSION

Of the three methods described for the cold alcohol fractionation of equine antitoxic plasma, Method 1 gives a product of definitely higher yield and slightly higher potency. However, the resulting antitoxin is unmodified by the enzyme digestion which is customarily considered to be beneficial in reducing the incidence of "serum sickness." Whether or not this enzyme digestion is really beneficial must be decided by extensive clinical comparisons of digested and undigested antitoxins of equal purity.

Of the two methods applicable to pepsin digested equine antitoxic plasma, Method 2 affords slightly higher recoveries and slightly higher purities than does Method 3. Method 3 is preferred, however, because of the much smaller volumes required.

The methods commonly used for the purification of antitoxic globulin employ fractionation with salts at elevated temperatures and require a dialysis for the eventual removal of salt. Such methods are not applicable to large-scale processing, and the temperatures employed are favorable for the formation of pyrogen. The cold alcohol methods, on the contrary, allow the preparation of antitoxin of very low pyrogenicity.

SUMMARY

1. The cold alcohol fractionation of heat-defibrinated antidiphtheria equine plasma was investigated within the ranges of *p*H 6.2 to 7.5, ethanol 16 per cent to 25 per cent, temperature 0° to −5°, and at ionic strength of approximately 0.08.

2. The cold alcohol fractionation of unmodified antidiphtheria equine plasma was investigated within the ranges of *p*H 5.1 to 7.4, ethanol 8 per cent to 35 per cent, temperature −2° to −5°, and at ionic strength of approximately 0.05.

3. The changes occurring during pepsin digestion of equine antitoxic plasma were investigated within the ranges of *p*H 2.4 to 4.0, ionic strength 0.02 to 0.08, and at a temperature of approximately 2°. The customary modification of the plasma proteins by pepsin was not obtained under these conditions at an ionic strength of 0.02, but was obtained at an ionic strength of 0.08.

4. The cold alcohol fractionation of pepsin digested antidiphtheria equine plasma was investigated within the ranges of *p*H 4.2 to 7.4, ethanol 5 per cent to 40 per cent, temperature −1° to −5°, and ionic strength 0.015 to 0.07.

5. Two methods are presented for preparing antitoxin of satisfactory yield and potency. One is applicable to unmodified antitoxic plasma and the resulting antitoxin is composed of the three antitoxin bearing globulins (γ, T, and β). The other is applicable to pepsin digested plasma and the resulting antitoxin is composed of essentially a single electrophoretic component.

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The Disintegration of Compressed Tablets*

By G. J. SPERANDIO,† R. V. EVANSON,† and H. G. DeKAY‡

A new type of apparatus for determining the disintegration rate of compressed tablets is described. Methods previously described are briefly reviewed. Data on results obtained by the new procedure are reported.

COMPRESSED tablets are one of the most popular forms of medication today, and both the United States Pharmacopœia and the National Formulary have admitted a variety of tablets as official preparations. The fact that the efficiency of a tablet is to a great extent influenced by the speed with which it disintegrates was recognized as early as 1902 (1), and in recent years both the British and the Swiss Pharmacopœias have included official methods for determining the time of disintegration for compressed tablets. However, in the United States, there is a variety of procedures currently in use for determining this factor, and there is a considerable discrepancy in the speeds with which different makes of the same type tablet disintegrate. All tablet manufacturers have disintegration tests which they apply to their tablets. According to a bulletin presented at a recent meeting of the American Pharmaceutical Manufacturers' Association, there are at least 12 different methods and types of apparatus currently in use for testing disintegration time (10).

The chief difficulty in determining disintegration time lies in the fact that different investigators interpret the word "disintegration" differently, frequently using solubility time for disintegration time; whereas in reality there should be a distinction between the two. In general, compressed tablets will do one of two things when put into a liquid: they will actually disintegrate, or fall apart, or they will slowly dissolve. The fact that a tablet disintegrates does not necessarily mean that it has dissolved; it is merely broken up into smaller particles. Consequently, in setting up standards for tablet disintegration, the fact that some types of tablets (usually those which do not contain a disintegrating agent) actually dissolve should be recognized.

The ideal definition of true disintegration time would be the time required for a tablet to break up into granules the size of the original granules from which it was compressed. However, manufacturing procedures vary so much that it would be difficult to establish a common size granule for all tablets; so in this investigation the granules in the above definition were considered to be of a size small enough to pass through an 8-mesh screen, since the majority of tablet granulations do not exceed that size.

This investigation was prompted by a desire to compare the most popular procedures now in use, and to discover, if possible, a simple method of testing disintegration time which would give consistent results with a minimum amount of equipment. If a standard method for determining the disintegration time of tablets could be accepted as satisfactory by all manufacturers, definite requirements could then be established for the official tablets, thereby insuring a uniformity of time of action as well as type of action.

Considering the fact that the principal testers of tablets are the industrial houses, those methods which by virtue of simplicity and/or compactness seemed most acceptable to use in an industrial laboratory were chosen for comparative tests. A brief description of these methods follows.

METHODS

The Industrial Method.—Various industrial firms who were contacted reported that they used with slight modifications the same method, which is known as the beaker and screen method. This consists simply of a wire screen of appropriate size supported in a beaker of water. The tablet to be tested is dropped onto the screen, which is then gently agitated. The time of disintegration is taken as the time required for the tablet to break apart and fall through the screen.

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The Modified Berry Method.—This type of apparatus consists of a beaker, over the top of which is a platform with a narrow opening in it. Through the opening a thread loop is placed over the tablet to be tested and on the end of the loop is a 50-Gm. weight. The beaker is filled with water and the tablet is allowed to rest over the opening in the platform, with the weight supported by it. The disintegration time is taken as the time required for the weight to break through the tablet and fall to the bottom of the beaker (5).

The A. O. A. C. Method.—Similar to the industrial method, this type consists of a wire gauze suspended in a beaker of water which also has an electric stirrer in it. The tablet is dropped into the gauze below the surface of the water, which is continuously agitated by the stirrer. The disintegration time is measured as the time required for the tablet to break apart and fall through the screen (6).

The Lewis Method.—This is a simple apparatus consisting of a piece of muslin stretched over the top of a small funnel, drum fashion, on top of which rests a flat spiral of wire about $^1/_{32}$ inch thick, on which the tablet is placed. At a height of 6 inches above the spiral is a jet from which one large drop of solvent falls on the tablet every second. The disintegration time is taken as the time required for the tablet to completely fall apart (2).

The É'we Method.—The principle of a glass tube of about 30 mm. inside diameter, the bottom of which is covered by a wire screen with a shallow depression in the center is used for the É'we Method. This tube is immersed in a beaker of fluid, the tablet is dropped into the tube, and a hog-bristle paint brush weighing about 30 Gm. is introduced into the tube so that the bristles rest on the tablet; then the brush is gently raised and lowered at the rate of 15 times per minute "until the tablet is dissolved or until it is disintegrated as shown by the breaking of the last portion of the tablet into at least several pieces" (3).

The Gershberg and Stoll Method.—This type of apparatus, which Gershberg and Stoll report as having been used by the laboratories of the U. S. Army Medical Department since 1940, basically consists of a rack containing glass tubes whose bottom openings are covered with wire screen. This rack is immersed in a beaker of water and a tablet is put in each tube. The rack is raised and lowered in the water at the rate of about 20 times per minute, and the disintegration time is taken as the time required for the tablets to break apart and fall completely through the screen (8).

The A. Ph. A. Method.—Proposed by Berry and modified by the A. PH. A. laboratory, this apparatus requires five test tubes which can be stoppered tightly and attached to a wheel or shaft which can be turned. This wheel is immersed in a constant temperature water bath, the tubes being filled with water, and a tablet is put into each tube. The tubes are stoppered and the wheel revolved slowly so that the tablets move gently from one end of the test tube to the other. The time required for the tablet to fall apart is taken as the disintegration time (7).

EXPERIMENTAL

Samples of competitive type tablets were obtained from seven different sources. Ten tablets from each lot were tested by each of the above methods, the time required for disintegration being checked with a stop watch. In order to standardize conditions, distilled water at room temperature was used as the liquid in all cases, and wherever a screen was required, a standard 8-mesh wire screen was employed. The average time required for disintegration or solubility of the ten tablets was calculated for each method, and is shown in Table I.

From the results presented in Table I, it can be seen that a considerable variation of disintegration time is obtained for the same tablet, using different methods; and that the greatest variation is in those tablets which have a tendency to dissolve rather than disintegrate, such as sodium salicylate and methenamine.

DISADVANTAGES OF METHODS TESTED

Each method tested was found to have certain disadvantages, which may be briefly summarized as follows:

The Industrial Method, while satisfactory for tablets which disintegrate quickly, is inconvenient for those which require a long time to disintegrate, and it is difficult for different operators to maintain the same speed of agitation of the screen. Also, this method is not practical for certain tablets which have a tendency to float on the surface of he liquid.

The Modified Berry Method, which is more of a stress test than one for disintegration, does not give a true disintegration time, since the suspended weight usually breaks the tablet in half; and certain tablets with transverse laminations resist the wire force since the weight does not permit them to swell properly and disintegrate normally. In many cases, these tablets contain hard cores which resist disintegration but do break under the pressure of the weight to give a false end point.

The A. O. A. C. Method has one chief drawback in that the current induced by the stirrer in the water has a tendency to draw the tablet up against the moving stirrer, which then breaks it up mechanically to give a false disintegration.

The Lewis Method is too time-consuming, since only one tablet can be tested at a time; and the force of the dropping water tends to push the tablet out of the path of the water so that it is no longer in the center of the dropping liquid.

The É'we Method, in our opinion, causes a false disintegration due to the unavoidable contact of the eccentric-geared brush with the tablet, which tends to push it through the screen.

The Gershberg–Stoll apparatus is cumbersome and makes an exact end point difficult to determine

TABLE I.—DISINTEGRATION TIME IN SECONDS

Company	Tablet	Industrial	Lewis	A. O. A. C.	Berry	Gershberg–Stoll	É'we	A. PH. A.
A	Aspirin, 5 gr.	26	29	19	18	26	29.1	22
	P. A. C.	46	51.3	48	45	54.3	61	52.4
	Sod. Bicarb., 5 gr.	15	26.8	19	5.5	12.7	13	28
	Atropine Comp.	132	129	135	75	131	135	160
	Ascorbic Acid	570	815	900	a	791	1680	850.3
B	Aspirin, 5 gr.	19.6	18.9	19.6	17.9	17.1	21.7	14.3
	Sulfasuxidine	26	28.3	27	21	22.1	41.4	28.2
	Sod. Salicylate, 10 gr.	1320	a	a	a	a	a	a
	Phenobarb., $1^1/_2$ gr.	15	10.9	15.2	9.2	7.8	11	8.6
C	Aspirin, 5 gr.	15.5	12.2	16	12.1	14	20	10.4
	Sulfathiazole	84	93	131	79.8	100	102	95
	Chloramine-T	210	190	504	588	390	651	365
	Ephedrine Comp.	635	643	598	705	785	546	888
	Sod. Salicylate, 5 gr.	390	540	a	960	858	1416	1280
	$KMnO_4$, 5 gr.	16	10	8	3.8	8.4	7	?
D	Aspirin, 5 gr.	7.6	14	40.3	12.8	15	9.5	16
	P. A. C.	186	274	383	518	285	397	479
	Thiamin HCl	216	260	378	760	370	402	354
	Sod. Bicarb., 5 gr.	54.9	49	69	110.6	85	110.6	84
	Ascorbic Acid	234	292	508	630	519	1202	572
	Sod. Salicylate, 5 gr.	450	481	585	782	844	965	672.5
E	Aspirin, 5 gr.	14.6	14	15.6	13.2	11.3	15.1	13
	Methenamine, 5 gr.	258	210	367	377.5	194	535	375
	Ca. Lactate, 5 gr.	240	270	284	214.8	156	271	270
	Sod. Bromide, 5 gr.	130	106	137	82.4	132	130	140
	P. A. C.	47.5	40	70	a	185	a	a
	P. A. C. Yellow	54.1	110	94	a	a	a	a
F	Aspirin, 5 gr.	5	11.2	4.8	5	7	7.1	6.9
	P. A. C.	9.6	18.4	14.1	8.4	11.5	18.7	28.8
	Acetanilid, 5 gr.	53.9	36.2	65	37.4	45.6	130.8	43.2
	Phenobarb., $1^1/_2$ gr.	133	112	111	135	105	137.4	113
	Bismuth Comp.	27	51.7	44	19.4	28	35	46
G	Aspirin, 5 gr.	10	9.8	29	8.9	17	11.6	15.1
	Sulfathiazole	104	110	105	68	119	122	94
	Phenacetin	43.2	37	105	45.4	51.2	32.5	70
	Mg. Trisilicate	33.6	25.7	79	17	26.2	29	29
	Ca. Lactate	25.1	38.4	26.8	5.4	12	22.8	35.5
	Ca. Carbonate	409	630	a	310	620	498	a
	Ascorbic Acid	60	101	86.6	86.6	58	96.3	115
	Thiamin	159	178	309	145	196	220	205
	Bi. Subgallate	135	94.9	176.2	69.6	110	101.8	130

a Tablets not completely disintegrated after thirty minutes.

because the disintegrating tablets tend to cloud up the solution which is churned by the up-and-down motion of the basket. Also, since the tubes are arranged in a circular rack, it is difficult to see them all clearly at the same time.

The A. PH. A. Method has two main objections: Too much time is required in getting the tablets in the tubes, stoppering them, and starting them in motion, and also in many cases turbid solutions are produced which make it difficult to see the end point.

Also, in the last three methods, the size of the tubes is such that many larger tablets will not fit into them and thus cannot be tested.

APPARATUS

Considering all factors involved in determining tablet disintegration, the ideal apparatus should, above all, be of simple construction so that standardized apparatus can be easily put together; it should be of such a nature that the tablets are continuously immersed in the testing liquid; the tablets should be kept in continuous gentle motion without being subjected to any unnatural stress; a definite means of establishing an end point should be specified; and the liquid in which the test is run should be kept clear so that the exact end point can be determined. With these requirements in mind, the following apparatus was designed as suitable for determining the disintegration time of tablets:

A circular cylinder $1^1/_2$ inches in diameter and $2^1/_2$ inches long was made from 8-mesh wire gauze. Each end of the cylinder was closed with 8-mesh wire disks, to the centers of which were soldered small axles, with a pulley wheel of the same diameter as the cylinder attached to one of the axles. A small trap door which could be opened and closed was made in the side of the cylinder, which was then suspended by the axles between two aluminum supports which had been built around a 1000-cc. beaker, so that the cylinder revolved freely on the axles. The pulley wheel was connected by a belt to another pulley attached to a small motor on a ring stand

TABLE II.—COMPARATIVE DISINTEGRATION TIME OF CERTAIN TABLETS

Company	Tablet	DeKay–Evanson–Sperandio Method Disintegration	Av. of Other Methods, Sec.
A	Aspirin, 5 gr.	26.2	24.1
	P. A. C.	56.5	51.1
	Sod. Bicarb., 5 gr.	18.7	17.1
	Atropine Comp.	127	128.1
B	Aspirin, 5 gr.	19	18.4
	Sulfasuxidine	28.5	27.7
	Phenobarbital, 1½ gr.	11	11.1
C	Aspirin, 5 gr.	13.8	14.3
	Sulfathiazole	114	97.8
	Chloramine-T	452	412.5
	Ephedrine Comp.	690	671.4
	Sod. Salicylate, 5 gr.	763	777.7
D	Aspirin, 5 gr.	14.4	16.4
	P. A. C.	394	360.3
	Thiamin, 50 mg.	385	391.4
	Sod. Bicarb., 5 gr.	84	80.4
	Ascorbic Acid	474	565.2
	Sod. Salicylate, 5 gr.	651	667
E	Aspirin, 5 gr.	12.7	13.8
	Methenamine, 5 gr.	307	330.9
	Ca. Lactate, 5 gr.	266	243 7
	Sod. Bromide, 5 gr.	118	122.5
F	Aspirin, 5 gr.	6.3	6.7
	P. A. C.	14.9	19.5
	Acetanilid, 5 gr.	69	68.8
	Phenobarbital, 1½ gr.	134	126.6
	Bismuth Comp.	30	35.8
G	Aspirin, 5 gr.	14	14.5
	Sulfathiazole	88	103.2
	Phenacetin	186.1	54.9
	Mag. Trisilicate	40	34.2
	Ca. Lactate	33	23.7
	Ca. Carbonate, 10 gr.	1105	493
	Ascorbic Acid	96	86.2
	Thiamin	204	201
	Bi-Subgallate	105	116.9

directly above the beaker. The beaker was then filled with distilled water at room temperature to a depth just sufficient to cover the top of the wire basket. To maintain a clear solution, an outlet tube was blown into the bottom of the beaker and an inlet tube was introduced into the top. Distilled water was then run in at the top and drained out at the bottom at a rate of 100 cc. per minute during the disintegration tests. The tablet or tablets were dropped into the trap door, which was then closed and the motor adjusted so that the basket rotated in the water at the rate of ten complete revolutions per minute. The apparatus is illustrated in Fig. 1. The time of disintegration was taken as the time required for the tablets to break apart into particles which would fall through the basket to the bottom of the beaker.

Each lot of tablets which had been tested by the preceding seven methods was then tested by the DeKay–Evanson–Sperandio Method and the average disintegration time for ten tablets from each lot was taken. For comparative purposes, this disintegration time is shown with the *average* disintegration time of the other seven methods combined. Table II.

It can be seen from Table II that the proposed method gives a disintegration time quite close to the average time of all the other methods tried in almost every case. There is a considerable variation in the case of the ascorbic acid tablets of Company D, and a very great difference in the case of the Phenacetin Tablets and Calcium Carbonate Tablets of Company G. It was found that in these cases where the disintegration time was much longer than average, it was because the granules into which the tablet broke up were of a larger size than the 8-mesh screen, and consequently had to disintegrate further before reaching the established end point.

As a further test for the efficiency of the DeKay–Evanson–Sperandio Method, it was decided to determine the disintegration time of the various samples of aspirin tablets, testing them one at a time and in increasing amounts up to five at a time, to see if consistent results could be obtained. These results are shown in Table III.

From this tabulation, it would seem that from one to five tablets can be tested at one time, depending on the wish of the operator, and that repeatable results can be obtained.

All tablets do not disintegrate at the same time given for a particular lot. Even though they may be stamped on the same machine, at the same time, at the same pressure, and from the same granulation, tablets will deviate from the average disintegration time. Table IV shows such deviation for a representative lot of tablets from various manufacturers. Since data for other methods can be found elsewhere, only the DeKay–Evanson–Sperandio Method will be considered here.

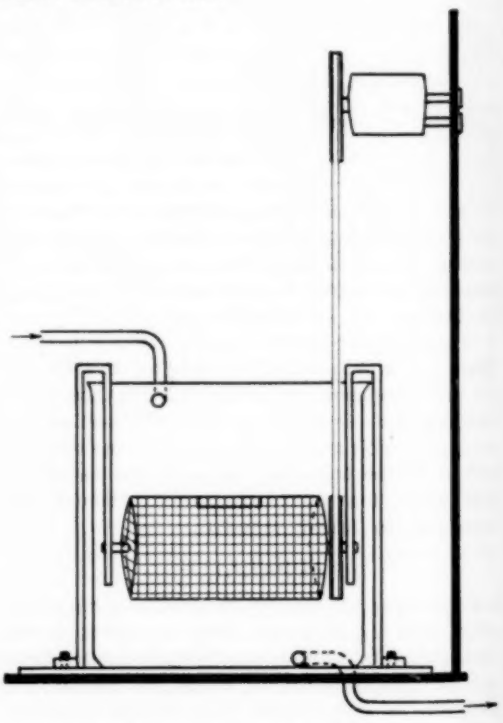

Fig. 1.—DeKay–Evanson–Sperandio Apparatus

TABLE III.—COMPARATIVE DISINTEGRATION TIME OF ASPIRIN TABLETS

No. of Tablets Tested at One Time	Disintegration Time, Sec.						
	Co. A	Co. B	Co. C	Co. D	Co. E	Co. F	Co. G
1	24	12	14	11	13	7	13.5
2	26.6	18	14.5	13	12.5	8	14
3	26	18	16	13.5	13	7	10.6
4	28	21	15.2	15	13.5	7.2	11.5
5	25.2	20	16.5	14	14	6.5	10
Av.	25.9	17.8	15.2	13.5	13.2	7.1	11.9

TABLE IV.—DISINTEGRATION TIME DEVIATION FOR A REPRESENTATIVE LOT OF TABLETS

Co.	Tablet	Disintegration Time, Sec.										Av.	Deviation, Sec.
B	Phenobarbital, 0.5 gr.	13	17	12	11	16	11	11	12	14	11	12.8	+4.2 −1.8
C	Sulfathiazole, 7.7 gr.	140	126	125	128	125	135	125	137	130	126	129.6	+10.4 −4.6
	Chloramine-T, 4.6 gr.	508	513	492	532	520	457	460	475	514	529	500	+32.0 −43.0
D	P. A. C.	439	306	313	408	345	339	422	430	365	355	372.2	+66.8 −66.2
	Sod. Bicarb., 5 gr.	72	114	88	45	148	122	51	58	55	57	80.8	+67.2 −35.8
	Thiamin HCl, 50 mg.	397	384	375	375	387	375	404	366	410	358	383.1	+26.9 −25.1
F	Aspirin Comp.	20	16	16	15	20	20	18	18	20	19	18.2	+1.8 −3.2
	Bismuth Subcarb.	33	36	33	32	33	35	31	33	34	33	33.3	+2.7 −2.3
	Phenobarbital, 1.5 gr.	117	189	115	203	180	135	190	191	190	175	132.5	+70.5 −17.5
G	Ascorbic Acid, 25 mg.	72	91	93	86	93	114	96	98	91	93	92.7	+21.3 −20.7
	Calcium Lactate 5 gr.	26.5	33	30.5	30	30.5	31	33	27	32	30	30.3	+2.7 −3.2
	Thiamin HCl, 5 mg.	260	292	263	270	252	245	273	251	241	247	259.4	+32.8 −14.4

SUMMARY AND CONCLUSIONS

Disintegration tests were run on a total of over 4000 tablets from seven different manufacturers, using seven different methods for determining the disintegration time.

It was found that no two methods consistently give the same average disintegration time for the same batch of tablets, and that some methods are not satisfactory for all types of tablets.

Several of the disintegration methods tried were deemed unsatisfactory because: (*a*) they did not have a definite end point which could be interpreted the same by all operators; (*b*) they produced a false end point either through stress or pressure on the tablet; (*c*) the end point was difficult to determine due to cloudiness or coloring of the solution.

A new method for determining disintegration time was devised and the various tablets were tested by it. The results obtained were found to compare favorably with the averages of the other methods combined.

We recommend this new method—the DeKay–Evanson–Sperandio Method—as an improved means of determining an accurate disintegration time for all types of tablets for the following reasons:

It gives a true disintegration time. The disintegration of the tablet is neither impeded nor accelerated by any physical force or unnatural shaking.

The end point is always definite and is the same for all tablets. The solution is kept clear, allowing the end point to be seen.

The disintegration times obtained are consistent for the same types of tablets.

From one to five tablets can be tested at one time.

All types of tablets can be tested.

It is simple to construct and occupies very little space.

Due to the many variable factors which influence the disintegration of compressed tablets, it would be necessary to make exhaustive tests and comparisons of all the accepted methods before one method could positively be established as preferable to all others; but it is inevitable that at some future time, a standardized method will be accepted officially. When this is done it will aid greatly in the production of uniform U. S. P. and N. F. tablets.

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A Study of the Physical and Chemical Properties of Natural Washington and Oregon Peppermint Oils*

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This report covers a four-year study of peppermint oil from various districts of Washington and Oregon. The following physical and chemical properties were ascertained for oils from each of the districts: total menthol, esters, optical rotation, specific gravity, refractive index, and solubility.

THE production of peppermint oil in the United States had its inception in 1816 in Wayne County, New York. In 1835 the cultivation of peppermint was introduced into Michigan and in a short period of time the industry spread into Indiana. About 1919 peppermint cultivation was initiated on the Pacific Coast and today this enterprise has developed into a large and flourishing industry. The abundance of suitable land, temperate climate, and an ample water supply are all contributing factors to the phenomenal growth of the peppermint industry in the states of Washington and Oregon.

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Main Oil-Producing Districts of Washington and Oregon

This study concerns four distinct types of peppermint oils, *viz.*, C, E, S, and K, depending upon the particular district in which the peppermint is grown. Following is a brief description of each district.

Type C.—The district producing this oil lies along the Washington and Oregon sides of the Columbia River approximately 40 miles from the ocean. The land has been diked and is drained by a series of ditches. Subirrigation is carried on during the growing season when necessary by raising the water level in the ditches. The soil is a combination of peat, river silt, clay loam, and black loam. The rainfall in this district is about 40 inches per year.

Type E.—This oil is produced along the upper Willamette and lower McKenzie rivers in the state of Oregon. The Willamette Valley at this point has an average rainfall of about 35 inches per year and the soil is a deep, sandy river silt. Water from creeks and small rivers is used in irrigating the peppermint by means of overhead sprinklers.

Type S.—The oil is obtained from peppermint grown on river silt and clay loam soil in the central section of the Willamette Valley in the state of Oregon. The average annual rainfall is about 33 inches and the mint land is irrigated by overhead sprinkler systems using water obtained from shallow wells.

Type K.—This oil is produced in eastern Washington, a dry region with an average annual rainfall of less than 6 inches. The soil is a rich, deep lava ash and requires irrigation.

EXPERIMENTAL

The lack of significant analytical data regarding the physical and chemical properties of natural peppermint oil produced in the four districts of Washington and Oregon previously mentioned in this paper provided a need for this study. Since the oils from the various localities are distinct in their odor and flavor, it was reasonable to assume that some of their physical and chemical constants would vary.

In most cases each sample is representative of a full drum (400 pounds) of oil and all the samples used in this investigation were from commercial lots of oil. To the best of our knowledge the age of these samples at the time of analysis varied from one to six months, the greater part were not over three months old when analyzed.

Constants and Methods Used

Esters.—The U. S. P. XII method was used and the esters were calculated as menthyl acetate.

Total Menthol.—The U. S. P. XII procedure and formula for calculating the per cent of menthol were employed.

Optical Rotation.—The optical rotation was obtained at 25° using a 100-mm. tube and a Rudolph routine polarimeter equipped with a General Electric sodium laboratory arc as the source of light.

Specific Gravity.—A 10-Gm. pycnometer with attached thermometer was used in determining the specific gravity (25/25°).

Refractive Index.—This constant was ascertained with an Abbe refractometer at 20°.

Solubility.—The procedure designated in the U. S. P. XII for dementholized or impure peppermint oil was used. A soluble oil was rated as one clearly soluble at 25° in 3 volumes of 70% alcohol, by volume, with no separation of oil globules.

RESULTS

The values obtained for the various physical and chemical constants of natural Washington and Oregon peppermint oils are presented in tabular form. The minimum, maximum, and average results are presented in Table I for each of the four types, together with the year the oil was produced and the number of samples analyzed.

Table II shows the solubility data of the several types of natural peppermint oils in 70% alcohol at 25°. As designated in the United States Pharmacopœia XIII, the soluble oils are clearly soluble in 3 volumes of 70% alcohol, by volume, with no separation of oil globules.

TABLE I.—SOME PHYSICOCHEMICAL CONSTANTS OF NATURAL WASHINGTON AND OREGON PEPPERMINT OILS

No. of Samples	Year	Type of Oil		Esters, %	Total Menthol, %	Optical Rotation, 100 Mm./25°	Specific Gravity, 25°/25°	Refractive Index, 20°
33	1942	C	Min.	4.0	43.9	−21.0	0.8974	1.4622
			Max.	7.3	61.2	−26.3	0.9032	1.4663
			Av.	5.1	52.5	−23.9	0.9025	1.4633
146	1943	C	Min.	2.9	42.5	−17.0	0.8966	1.4606
			Max.	8.7	64.3	−31.0	0.9161	1.4675
			Av.	4.8	51.9	−23.4	0.9007	1.4624
27	1943	E	Min.	3.6	47.0	−20.0	0.8969	1.4610
			Max	11.2	66.2	−27.0	0.9131	1.4648
			Av.	6.4	58.3	−23.2	0.9025	1.4635
104	1943	K	Min.	3.0	38.9	−7.0	0.8812	1.4630
			Max.	9.9	64.0	−22.2	0.9190	1.4680
			Av.	5.7	53.2	−16.3	0.9144	1.4655
57	1944	C	Min.	3.53	45.64	−18.0	0.8995	1.4613
			Max.	11.27	66.44	−29.2	0.9141	1.4672
			Av.	5.90	55.65	−22.7	0.9031	1.4632
10	1944	S	Min.	4.07	49.07	−18.0	0.9004	1.4620
			Max.	9.66	62.19	−26.8	0.9050	1.4653
			Av.	7.36	57.43	−22.6	0.9032	1.4636
213	1944	K	Min.	3.60	36.63	−9.0	0.9017	1.4618
			Max.	16.26	72.07	−22.5	0.9519	1.4712
			Av.	6.36	52.47	−15.2	0.9084	1.4653
101	1945	C	Min.	4.12	42.87	−15.5	0.8993	1.4612
			Max.	12.00	67.06	−29.0	0.9135	1.4670
			Av.	6.74	56.25	−24.1	0.9024	1.4632
108	1945	E	Min.	5.03	52.23	−19.8	0.8979	1.4610
			Max.	14.80	69.91	−29.7	0.9078	1.4647
			Av.	7.86	59.44	−25.1	0.9019	1.4618
58	1945	S	Min.	4.50	47.20	−17.7	0.8998	1.4618
			Max.	11.50	64.99	−27.4	0.9286	1.4681
			Av.	7.36	56.98	−23.1	0.9032	1.4632
202	1945	K	Min.	3.94	39.15	−10.4	0.8990	1.4622
			Max.	10.96	65.21	−26.0	0.9271	1.4672
			Av.	6.65	53.60	−18.5	0.9057	1.4646

DISCUSSION

Tabulated below are the U. S. P. standards of peppermint oil and the average results of each type of natural oil for the years in which the investigations were made as designated in Table I above.

In Table I the maximum and minimum figures listed represent extreme values for the various constants investigated and are not representative of the true value of the various oils. Therefore for comparison with the U. S. P. standards only the average figures are considered. From the above table it is apparent that the average ester and menthol content of the four types of natural peppermint oils meet the U. S. P. standards. Likewise the optical rotation, specific gravity, and refractive index of types C, E, and S natural oils conform to the pharmacopœial requirements. However type K with an average rotation of −16.7 (U. S. P. −18 to −32) is lower than the official standard. Furthermore the specific gravity of type K, 0.896 (U. S. P. 0.896 to 0.908) and refractive index, 1.4651 (U. S. P. 1.4590 to 1.4650) are slightly higher than the official limits. One must bear in mind that all of these values are based on natural oils and under normal rectification there is an alteration to a certain extent of the constants in redistilled oils.

Preliminary studies indicate that the specific gravity and refractive index of type K peppermint oil will fall within the U. S. P. limits upon rectification. However, the optical rotation is not sufficiently altered by this treatment to conform to the standards of the Pharmacopœia.

The investigation of Washington and Oregon peppermint oils is being continued in the Pharmaceutical Chemistry Laboratories at the University of Washington.

TABLE II.—SOLUBILITY OF NATURAL WASHINGTON AND OREGON PEPPERMINT OILS IN 70% ALCOHOL

Type of Oil	No. of Samples	Soluble, %	Insoluble, %
C	337	95.41	4.59
E	135	97.04	2.96
S	68	98.55	1.45
K	519	85.00	15.00

TABLE III.—COMPARISON OF THE AVERAGE PHYSICOCHEMICAL CONSTANTS OF THE VARIOUS TYPES OF NATURAL PEPPERMINT OILS WITH THE STANDARDS OF U. S. P. XIII PEPPERMINT OIL

Type of Oil	No. of Samples	Ester, %	Total Menthol	Optical Rotation, 100 Mm./25°	Specific Gravity 25°/25°	Refractive Index, 20°
C	337	5.60	53.90	−23.5	0.9018	1.4629
E	135	7.57	59.21	−24.7	0.9020	1.4621
S	68	7.36	57.05	−23.03	0.9032	1.4633
K	519	6.34	53.06	−16.7	0.9086	1.4651
U. S. P.	...	Not less than 5	Not less than 50	−18 to −32	0.896 to 0.908	1.4590 to 1.4650

SUMMARY

1. The results of a study on the physical and chemical constants of natural peppermint oil from four districts of Washington and Oregon are reported.

2. A study of the solubility characteristics of these four oils is included.

WHO MAKES IT?

The National Registry of Rare Chemicals, Armour Research Foundation, 33rd, Federal and Dearborn Streets, Chicago, Ill., seeks information on sources of supply for the following chemicals:

1,1,1-Trichloro-2,2-bis(*p*-aminophenyl)ethane
3-Geranylcatechol
Catechol monophenyl ether
5-Vinylguaiacol
5-Allylguaiacol
3-Methyl-4-thioquinazolone-1,3
Maltobionic acid
2-Propenylpyridine
l-Tartaric acid
Embden ester
Chlorogenine
Digitogenine
Benzoxanthene
17-Hydroxycorticosterone
11-Dehydro-17-hydroxycorticosterone
17-Hydroxy-11-desoxycorticosterone
Pregnene-4-triol-17(β),20(β),21-dione-3,11-monoacetate-21
Pregnene-4-triol-17(β),20(β),21-dione-3,11-diacetate-20,21
Pregnene-4-diol-17,20-one-3-al-21

Adjusted Collyria Containing Zinc Salts*

By WILLIAM J. HUSA† and JACK K. DALE‡

Experiments on complex compounds of zinc led to the development of zinc collyria from which the zinc is not precipitated at *p*H 7.4, which is the approximate *p*H value of the tear secretion. It was found that citrates, tartrates, and glycine may be added to borate buffers to produce stable zinc collyria with *p*H values as high as 8.7. A new barbital-sodium barbital buffer has been devised which has maximum buffer capacity at the approximate *p*H of the tear secretion.

DILUTE solutions of zinc salts, with or without other medicaments, are widely used as ophthalmic astringents. To prevent precipitation of the zinc by the alkalinity of the tear secretion, it is common practice to make the solution acid by use of boric acid or acid buffer solutions.

The purpose of the present investigation was to develop collyria from which the zinc is not precipitated at *p*H 7.4, which is the approximate *p*H value of the tear secretion. This work involved a further study of buffers and led to the preparation of a new barbital-sodium barbital buffer which has maximum buffer capacity at the approximate *p*H of the tear secretion.

Collyria containing zinc have been used since ancient times. A zinc sulfate collyrium was included in an American military pharmacopœia in 1781 (1). The U. S. P. of 1820 contained formulas for collyria of zinc acetate and zinc sulfate (2). Various articles concerning precipitates in solutions of zinc salts appeared during the nineteenth century; these precipitates were usually removed by filtration. One of the first attempts to insure solution of all the zinc is found in the formula for zinc sulfate solution of the Edinburgh pharmacopœia, in which a small proportion of dilute sulfuric acid was used (3).

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The problem of preparing zinc collyria free of precipitated basic salts was not very successfully solved until 1918, when Peacock and Peacock (4) showed that precipitation in zinc chloride collyria could be prevented by use of boric acid and ammonium chloride.

H. Lipschutz (5) in 1929 demonstrated that the alkalinity of the tears was sufficient to precipitate all the zinc from an unbuffered 0.33% zinc sulfate collyrium. He first recommended an acetate buffer of *p*H 5.17 but later suggested the use of 0.5% to 1% of ammonium chloride.

Weakly buffered boric acid solutions and borate buffers with *p*H values from 4.2 to 6 have been recommended as suitable vehicles for zinc salts (6–9).

An isotonic zinc collyrium buffered and stabilized with boric acid and sodium acetate at *p*H 6.75 was introduced by Arrigoni, Fischer, and Tozer in 1941 (10); this collyrium contained methyl and propyl *p*-hydroxybenzoates as preservatives against molds and fungi.

Bijleveld and Dekking (11) in 1941 found that the addition of sodium citrate permitted the *p*H of borate buffered zinc collyria to be raised to 6.2 or higher without precipitation. In the same year Vogelenzang (12) published a formula for a similarly stabilized zinc sulfate collyrium having a *p*H of 7.2–7.4 and a freezing point of −0.80°.

EXPERIMENTAL

Barbital-Sodium Barbital Buffer Mixtures.—The practical usefulness of a weak acid in the preparation of a graduated series of buffer solutions is limited to a range of about 4 *p*H units. In general, the best buffer capacity is obtained when the concentrations of the acid and salt are approximately equal. In solutions containing equimolar concentrations of a weak acid and its salt, the negative logarithm of the ionization constant of the acid becomes numerically equal to the *p*H, i.e., $pK_a = pH$ under these conditions. Since the pK_a of boric acid is 9.24, the useful range of borate buffers is from about 7.24 to 11.24. Since borate buffers are the basis of Gifford's and Feldman's buffer solutions, it follows that these solutions are somewhat lacking in buffer capacity in the range of *p*H 6.6–7.8. Hence an effort has been

made in the present investigation to develop a buffer system having better buffer capacity in the range *p*H 6.6–7.8 which is needed in ophthalmic practice.

A search was made for other chemicals with maximum buffer capacity in the vicinity of *p*H 7.4. Phosphate buffers with a pK_{a2} of 7.21 adequately cover the desired *p*H range but they are incompatible with many ophthalmic prescription ingredients, notably zinc and silver salts. Theoretically *p*-hydroxybenzoic acid should have maximum buffer capacity at *p*H 6.94 but this compound is too insoluble for practical use. Diethylbarbituric acid (Barbital, U. S. P.) has a pK_a value of 7.43 (13) and appeared promising although the slight solubility necessitates the use of 0.02 *M* solutions which are about one-tenth as concentrated as the usual borate buffers and hence have less buffer capacity.

A mixture of equal volumes of 0.02 *M* barbital, U. S. P., and 0.02 *M* sodium barbital, U. S. P., was prepared in order to compare the pK_a value with the actual *p*H value. The latter was found to be *p*H 7.93 at 26° as compared with the usually accepted pK_a value of 7.43. A *p*H value of 7.95 at 18° was reported by Britton and Robinson (14).

All *p*H determinations were made with the Beckman glass electrode *p*H meter, laboratory model G, using 5-inch external type glass electrodes. The Beckman alkali resistant glass electrode was used for determinations above *p*H 9. The *p*H meter was set at the temperature of the solution to be measured. Before use and after each ten *p*H determinations the *p*H meter was calibrated, using a 0.05 *M* potassium acid phthalate solution prepared from a standard salt supplied by the National Bureau of Standards. This solution has a *p*H of 4.01 at 25°.

The results of *p*H determinations on various barbital buffer mixtures are given in Table I.

The results in Table I indicate that the addition of 1% of sodium chloride lowered the *p*H of the barbital buffers. The addition of sodium citrate lowered the *p*H above 7.3 and increased the *p*H below 7.3. This effect illustrates the principle that neutralization of a mixture of two weak acids having *p*H values within several units of each other gives a *p*H curve which is approximately a straight line over the buffer range of both acids.

It is generally accepted that the activity of the hydrogen ion increases with increasing temperature, i.e. the *p*H of a solution drops as the temperature is raised. The results in Table I indicate that for modified buffer C, a temperature rise of one degree lowers the *p*H an average of 0.03 *p*H unit over the range 27° to 37°. Modified buffer C appears suitable for use over the *p*H range of 6.6 to 7.8 which is desired in ophthalmic practice.

TABLE I.—*p*H VALUES OF BARBITAL-SODIUM BARBITAL BUFFER MIXTURES

Buffer Mixture		*p*H Values				
Cc. 0.02 *M* Barbital	Cc. 0.02 *M* Sodium Barbital	Plain Buffer at 27°	Modified Buffer A[a] at 27°	Modified Buffer B[b] at 27°	Modified Buffer C[c] at 27°	Modified Buffer C[c] at 37°
0	10	9.76	9.55	9.39	9.52	9.04
1	9	8.83	8.65	8.71	8.69	8.35
2	8	8.50	8.33	8.40	8.38	8.02
3	7	8.27	8.11	8.19	8.18	7.82
4	6	8.06	7.92	8.00	7.98	7.63
5	5	7.91	7.76	7.83	7.80	7.48
6	4	7.73	7.59	7.66	7.61	7.30
7	3	7.55	7.39	7.49	7.42	7.12
8	2	7.33	7.15	7.30	7.22	6.92
9	1	6.99	6.80	7.09	6.95	6.70
10	0	5.13	5.01	6.83	6.60	6.41

[a] Plain buffer + 1% sodium chloride.
[b] Plain buffer + 0.5% sodium citrate.
[c] Plain buffer + 1% sodium chloride and 0.5% sodium citrate.

TABLE II.—PROPORTIONS OF ACID AND ALKALINE BUFFERS REQUIRED IN PREPARING MODIFIED BUFFER C

Desired *p*H Value	Cc. Acid Buffer Solution[a]	Cc. Alkaline Buffer Solution[b]
6.6	9.3	0.7
6.7	9.0	1.0
6.8	8.6	1.4
6.9	8.1	1.9
7.0	7.7	2.3
7.1	7.2	2.8
7.2	6.6	3.4
7.3	6.0	4.0
7.4	5.5	4.5
7.5	4.9	5.1
7.6	4.2	5.8
7.7	3.6	6.4
7.8	3.1	6.9

[a] 0.02 *M* barbital solution containing 1% of sodium chloride and 0.5% of sodium citrate.
[b] 0.02 *M* sodium barbital solution containing 1% of sodium chloride and 0.5% of sodium citrate.

The quantities of acid and alkaline buffers necessary to give *p*H values from 6.6 to 7.8 at 37°, with intervals of 0.1 *p*H unit, were determined by interpolation of a graph based on the first and last columns of Table I. The *p*H values were checked experimentally and agreed with the interpolated values within 0.02 *p*H unit. The results are given in Table II.

Effect of Zinc Salts in Buffer Mixtures.—It is known that the *p*H at which a metallic hydroxide precipitates depends to some extent on the anions present. Hence different zinc salts were used in

Gifford's (8) and Feldman's (9) buffer solutions to determine which salts would give the least precipitation. Solutions of zinc acetate, bromide, chloride, iodide, nitrate, phenolsulfonate, and sulfate were prepared in 0.01 *M* concentration. Each of these solutions was mixed with an equal volume of a series of double-strength Gifford's buffer mixtures. Identical experiments were carried out with double-strength Feldman's buffer mixtures. The resulting mixtures contained zinc salts in 0.005 *M* concentration in buffer solutions covering the *p*H range of 5 to 7.4 with intervals of approximately 0.2 *p*H unit. All of the zinc salts gave clear solutions with both buffer systems up to and including *p*H 6.8, except for the bromide and chloride, which gave precipitates of basic salts over the entire range. Slight precipitates appeared at *p*H 6.99 with the acetate and sulfate in Gifford's solution and with the acetate in Feldman's solution. Heavy precipitates indicative of zinc hydroxide formation appeared in solutions of all salts in both buffer systems at *p*H 7.19 and 7.35. After three days nearly every solution showed at least a slight amount of flocculent precipitate which on microscopic examination showed cellular structures which appeared to be molds and yeasts as previously reported by Skauen and Burroughs (15). Traces of basic salts were found in mixtures of *p*H 6 with more noticeable amounts above *p*H 6.4. Zinc sulfate and nitrate gave the slightest amount of basic salt formation on standing.

Zinc sulfate gave precipitates at *p*H 6.8 and above in barbital-sodium barbital buffers containing no sodium citrate, the concentration of zinc sulfate being 0.0075 *M*. In presence of 0.5% sodium citrate, as in modified buffers B and C, the solutions of zinc sulfate remained clear over the *p*H range 6.6 to 9.5.

A search of the literature indicated that the following compounds have been reported as preventing precipitation of zinc salts in neutral or alkaline solution: sodium citrate (11, 12, 16, 17), sodium acetate (10), sodium tartrate (18), ammonium chloride (4, 5, 19), aminoacetic acid (20), and *o*-, *m*-, and *p*-aminobenzoic acids (21).

Alkaline Zinc Collyria.—Tests were made with various substances to obtain clear, alkaline zinc solutions suitable for use as collyria. The quantities of acid and alkaline buffer substances required to give the desired *p*H were obtained by interpolation of neutralization curves of the acid and alkaline buffer solutions, each of which contained the same concentration of zinc salt and stabilizing agent and sufficient neutral salt or other compound to attain approximate isotonicity with the tear secretion. The quantity of substance required to make the solutions isotonic was calculated by the osmotic factor method (22). In each case the freezing point was determined by means of the Bartley apparatus (23). The formulas which follow appear satisfactory for ophthalmic use on the basis of *p*H and isotonicity but clinical tests are needed to determine how the alkaline zinc collyria compare therapeutically with presently used zinc collyria which are slightly acid in reaction. All the formulas are stated in terms of the number of grams of each solute in 100 cc. of solution.

Formula 1

Barbital	0.15
Sodium Barbital	0.25
Sodium Citrate	0.50
Sodium Chloride	1.00
Zinc Sulfate	0.25
Distilled Water, *q. s.*	100.00

Formula 1: *p*H 7.48 at 28°. F. p. = −0.78°. Gave no sensation in eyes. No precipitation after one month. Mold growth abundant.

Formula 2

Barbital	0.26
Sodium Barbital	0.12
Sodium Citrate	3.18
Zinc Sulfate	0.25
Distilled Water, *q. s.*	100.00

Formula 2: *p*H 7.40 at 27°. F. p. = −0.65°. Gave no sensation in eyes. Remained clear even after several months.

Formula 3

Barbital	0.26
Sodium Barbital	0.19
Potassium Sodium Tartrate	4.14
Zinc Sulfate	0.25
Preserved Distilled Water	100.00

Formula 3: *p*H 7.38 at 25°. F. p. = −0.74°. Gave a slight stinging sensation in eyes. Mold growth in seven days, no other precipitation after several months.

The preserved distilled water was made by dissolving 0.026% of methyl *p*-hydroxybenzoate and 0.014% propyl *p*-hydroxybenzoate in distilled water.

Formula 4

Ammonium Chloride	0.60
Sodium Barbital	0.75
Sodium Chloride	0.48
Zinc Sulfate	0.25
Preserved Distilled Water	100.00

Formula 4: *p*H 7.52 at 28°. F. p. = −1.09°. Gave a slight stinging sensation in eyes. Remained clear even after several months.

Formula 5

Hydroxylamine Hydrochloride	0.25
Sodium Barbital	1.48
Potassium Chloride	0.78
Zinc Sulfate	0.25
Preserved Distilled Water	100.00

Formula 5: *p*H 7.49 at 28°. Remained clear for several weeks.

Formula 6

Aminoacetic Acid	0.15
Sodium Barbital	0.41
Sodium Chloride	1.10
Zinc Sulfate	0.25
Preserved Distilled Water, *q. s.*	100.00

Formula 6: *p*H 7.33 at 28°. F. p. = −0.78°. Gave no sensation in eyes. Remained clear even after several months.

Formula 7

Aminoacetic Acid	0.15
Sodium Barbital	0.30
Dextrose	7.73
Zinc Sulfate	0.125
Preserved Distilled Water, *q. s.*	100.00

Formula 7: *p*H 7.59 at 25°. F. p. = −0.85°. Gave no sensation in eyes. Remained clear on standing several months.

Formula 8

Citric Acid	0.54
Sodium Borate	1.70
Zinc Sulfate	0.25
Potassium Chloride	0.86
Preserved Distilled Water, *q. s.*	100.00

Formula 8: *p*H 7.60. F. p. = −0.88°. Gave slight stinging sensation in eyes. Stable after several months.

Formula 9

Aminoacetic Acid	2.12
Sodium Borate	0.63
Boric Acid	0.83
Zinc Sulfate	0.25
Preserved Distilled Water, *q. s.*	100.00

Formula 9: *p*H 7.58 at 25°. F. p. = −0.91°. Gave no sensation in eyes. Stable after several months.

Formula 10

Aminoacetic Acid	0.30
Sodium Borate	1.50
Dextrose	4.16
Zinc Sulfate	0.25
Preserved Distilled Water, *q. s.*	100.00

Formula 10: *p*H 7.44 at 26°. F. p. = −0.77°. Gave no sensation in eyes. Remained clear even after several months.

DISCUSSION

Several of the formulas yield solutions having a *p*H of about 7.6 at room temperature but these *p*H values drop to about 7.4 at body temperature.

In Formulas 4 to 7, inclusive, no barbital is included in the formula since the acidity of aminoacetic acid, ammonium chloride and hydroxylamine hydrochloride, respectively, liberates sufficient barbital to give the desired buffer action. In Formulas 2, 3, and 9 the stabilizer is used in sufficient quantity to act also as an isotonic adjusting agent.

Formulas 8 to 10, inclusive, comprise borate buffers in which the zinc is kept from precipitating in alkaline solution by the presence of citric acid or aminoacetic acid. Formula 10 illustrates the ability of dextrose to increase the ionization of boric acid so that it becomes a stronger acid, as the addition of dextrose reduced the *p*H from 8.3 to 7.4.

SUMMARY

1. A barbital-sodium barbital buffer was developed as a possible ophthalmic buffer having maximum buffer capacity at the approximate *p*H of the tear secretion.

2. Formulas have been developed for solutions of zinc salts in which the use of stabilizers, such as citrates, tartrates and aminoacetic acid, prevents the precipitation of zinc at *p*H 7.4, which is the approximate *p*H of the tear secretion.

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The Analysis of Ipecac—Drug, Tincture, and Fluidextract*

By FLOYD A. WHIPPLE and J. M. WOODSIDE

Several modifications of the official methods of assay for ipecac and ipecac preparations have been devised and tested. These modifications are described and the results obtained by each are recorded. A general method of assay, applicable to ipecac and ipecac preparations and with fewer inherent sources of error than those of any of the official methods, is recommended.

IPECAC and its preparations have had official use in medicine for many years. The present methods of assay have remained practically the same for the last several revisions of the U. S. P. and N. F.

However, different operators (1), at times, have expressed various difficulties in the extraction of the alkaloids by the official methods, especially with the N. F. tincture. The U. S. P. XIII method (2) for the drug and fluidextract involves, as do certain other official assays, the transfer of an aliquot part of a very volatile solvent, ether. In this method, 10 Gm. of drug or 10 cc. of the F.E. absorbed on filter paper or asbestos is macerated overnight in 100 cc. of ammonia-ether solution. After considerable shaking and allowing to stand overnight, a 50-cc. aliquot of the ammonia-ether solution (very difficult to measure accurately!) is then taken, which theoretically represents 5 Gm. of drug or 5 cc. of the F.E. Even when this operation is carried out as rapidly as possible, and at controlled temperatures, it seems that some of the ether cannot help but be vaporized in the shaking process, and therefore the ether solution obtained will be somewhat concentrated. The method of the A.O.A.C. (3) avoids this by making an acid dilution and taking the aliquot part of the acid solution and not of an ether solution of the alkaloids. Digangi and Trupp (1) also suggested preparation of an acid filtrate for apparently the same reason. They expressed the fact that the difficulty with the N. F. tincture (4) method is mostly from a manipulative standpoint. We, too, usually find it practically impossible to obtain a satisfactory result in this case without enduring much emulsification and using ether in a volume too great for practical purposes. This prompted the running of several "research" experiments—using the official assay methods and then applying some of the suggested modifications to see if a method of assay could be obtained which is more readily workable and whereby check results can be obtained.

* Received Sept. 5, 1947, from the Chemical Control Laboratories, Eli Lilly and Company, Indianapolis, Ind.

EXPERIMENTAL

I. Ipecac (Drug)

(a) **Soxhlet Extraction.**—Place 5 Gm. of the drug in an extraction thimble, insert the thimble in a Soxhlet extractor, as directed in the U. S. P. XIII, page 677, paragraph "C" and, after macerating with ammonia T. S. overnight, extract with ether for four hours, then continue as in the U. S. P. XIII method, with the acid extractions.

(b) **Percolation.**—Place 5 Gm. of the drug in a small percolator. Macerate overnight with ammonia T. S., then percolate the next day with ether until the drug is exhausted, and continue as in the U. S. P. XIII method, extracting the ether with acid, etc.

(c) **Maceration.**—Macerate 5 Gm. of the drug in a glass-stoppered flask overnight using ammonia T. S. and 50 cc. of ether. Shake the mixture for one hour, then decant the ether through a filter. Wash the residual drug with several portions of ether, adding these through the filter. Combine the total ether extracts, evaporate to about 50 cc., and continue the assay as in the U. S. P. XIII, extracting the ether with acid, etc.

(d) **U. S. P. XIII Method.**—(As is.)

SERIES NO. I.—RESULTS OF IPECAC ASSAYS BY METHODS DESCRIBED ABOVE

Drug No. 1

	Method	Result, %	Check Result, %
(a)	Soxhlet	2.65	2.60
(b)	Percolation	2.40	...
(c)	Maceration	2.58	2.66
(d)	U. S. P. XIII	2.75	2.88

Comments.—(a) The Soxhlet method works very well with no manipulative difficulty encountered.

(*b*) The Percolation method needs a large volume of ether and requires a longer time period than the others; also more cumbersome.

(*c*) The Maceration method is not too practical. The results check with the Soxhlet method, although the method of extraction is not as easy as in the Soxhlet method.

SERIES NO. II.—RESULTS OF IPECAC ASSAYS

Method	Operator No. 1, %	Operator No. 2., %
Drug No. 2		
(*a*) Soxhlet	2.95	2.88
	2.89	
(*b*) U. S. P. XIII	3.52	3.09
	3.18	
Drug No. 3		
(*a*) Soxhlet	2.79	2.86
	2.85	2.89
(*b*) U. S. P. XIII	2.97	3.03

Comments.—The results show that the Soxhlet method is superior to the U. S. P. XIII method in that it is easier to manipulate, the results seem more readily reproducible, and it is subject to no apparent source of error.

Proposed Assay Method for Ipecac Drug

Accurately weigh 5 Gm. of the ground sample, place it in an extraction thimble and insert the thimble in a suitable Soxhlet extractor. Add enough ether to thoroughly cover the drug and allow to stand five minutes, stirring occasionally. Add 3 cc. of stronger ammonia T. S. and mix thoroughly. Allow to macerate overnight, then cover the drug with a pledget of cotton, pack thoroughly, and extract with ether for four hours, or until completely extracted as shown by a suitable test.

Transfer the ether extracts to a separatory funnel, rinse the container with a small amount of ether, and continue as in the U. S. P. XIII assay method, page 278, second paragraph, beginning with "Completely extract the alkaloids," etc.

II. Fluidextract of Ipecac, U. S. P. XIII

(a) **N. F. VIII Method for Tincture Applied to F.E. (Triple Shake-Out).**—Here the N. F. VIII Tincture Ipecac method was followed using a 5-cc. sample and not evaporating, but adding 5 cc. of water.

(b) **Acid Filtrate Method** (1).—Dilute 10 cc. of F.E. with 10 cc. of dilute sulfuric acid and add enough distilled water to make exactly 100 cc. After filtering, transfer exactly 50 cc. of this dilution to a separatory funnel and extract twice with ether. Combine these ether extracts and wash with 10 cc. of distilled water. Add the water to the acid solution and discard the ether. Add enough ammonia to make the solution alkaline, then extract with 50-cc. portions of ether, evaporate these, and titrate the alkaloids in the usual manner.

(c) **Modification of the N. F. VIII Method.**—Dilute 5 cc. of F.E. with 50 cc. of distilled water in an automatic extraction apparatus (Palkin-Watkins), add 5 cc. of ammonia T. S., and extract with ether for four hours. Carefully transfer the ether to a separatory funnel, extract with acid, and continue as in the U. S. P. method.

(d) **U. S. P. XIII Method.**—Run as in U. S. P. XIII.

SERIES NO. III.—RESULTS OF FLUIDEXTRACT ASSAYS

Method	Result (Gm. Alkaloids per 100 Cc.)	Check Result (Gm. Alkaloids per 100 Cc.)
F.E. No. 1		
(*a*) Triple shake-out (N. F. VIII)	2.11	1.84
(*b*) Acid filtrate	2.40	2.26
(*c*) Modified N. F. VIII	2.13	2.17
(*d*) U. S. P. XIII	1.91	2.20

Comments.—(*a*) The triple shake-out method is long and tedious. Several emulsions were experienced.

(*b*) The acid filtrate method is more readily workable but not in line with any comparable method for the drug. The results are higher than by other methods, probably because there is no preliminary series of ether shake-outs.

SERIES NO. IV.—RESULTS OF FLUIDEXTRACT ASSAYS

Method	Result (Gm. Alkaloids in 100 Cc.) Operator No. 1	Check Result (Gm. Alkaloids in 100 Cc.) Operator No. 2
F.E. No. 2		
Modified N. F. VIII	2.086	2.11
U. S. P. XIII	2.16	2.04

Comments.—We believe the modification of the N. F. VIII Tincture method, using automatic extraction for the first series of alkaline-ether shake-outs, is a method superior to the method of the U. S. P. XIII for the F.E. because:

(*a*) It eliminates taking an aliquot part of a volatile solvent, ether.

(*b*) The material does not have to stand overnight and an assay can be run in about five hours (over-all time), actually using less than an hour of the analyst's time.

(*c*) It gives more concordant results.

(*d*) It eliminates absorption of the F.E. on filter paper.

(*e*) It is very easy to manipulate.

Suggested Revision Method for the Assay of Fluid Extract Ipecac, U. S. P.

Place exactly 5 cc. of Fluidextract Ipecac in a Palkin–Watkins automatic extractor and add 50 cc. of distilled water. Make the solution in the extractor distinctly alkaline with stronger ammonia water and carefully add the proper amount of peroxide-free ether. Connect the extractor to a condenser and extract for four hours. Occasionally disconnect the extractor from the condenser and agitate the lower layer by raising and lowering the center extraction tube. After the four-hour extraction period, disconnect the extractor and carefully transfer the ether to a 250-cc. separator. Wash the extraction flask with two small portions of ether and add these to the separator. Extract the alkaloids from the ether solution by shaking with four 25-cc. portions of 2% sulfuric acid. Now make the acid extractions alkaline by adding a slight excess of stronger ammonia and extract with five 40-cc. portions of peroxide-free ether. Filter the ether into a 250-cc. beaker. Evaporate until the volume is about 2 cc., add 5 cc. alcohol and 20 cc. 0.05 *N* H_2SO_4. Warm on the steam bath for a few minutes to expel the last traces of ether, then cool and titrate with 0.02 *N* NaOH, using Methyl Red as the indicator.

Each cc. 0.02 *N* H_2SO_4 = 4.80 mg. Ipecac alkaloids.

III. Tincture of Ipecac N. F. VIII

(*a*) The acid filtrate method [as described by Digangi and Trupp (1)].

(*b*) The U. S. P. XIII F.E. method modified in that 50 cc. of the Tincture is used as a sample.

(*c*) The N. F. VIII method modified as in the assay of the F.E., using automatic extraction for the first series of alkaline-ether shake-outs.

(*d*) N. F. VIII as prescribed.

SERIES NO. V.—RESULTS OF TINCTURE ASSAYS

Method	Result (Gm. Alkaloids per 100 Cc.)	Check Result (Gm. Alkaloids per 100 Cc.)
(*a*) Acid filtrate	0.245	0.232
(*b*) Modified U. S. P. XIII	0.228	0.145
(*c*) Modified N. F. VIII	0.211	0.205
(*d*) N. F. VIII	0.107	0.185
		0.201

Comments.—(*a*) By the acid filtrate method the results were unusually high.

(*b*) The modified U. S. P. XIII method had too much liquid to be properly absorbed; seemingly not practical for Tincture.

(*c*) The modified N. F. VIII method was easiest to work—no emulsification and good duplication of results.

SERIES NO. VI.—RESULTS OF TINCTURE ASSAYS
Tincture No. 2

Modified N. F. VIII Method

	Gm. Alkaloids per 100 Cc. Operator No. 1	Gm. Alkaloids per 100 Cc. Operator No. 2	N. F. VIII Method as Is
Sample No. 1	0.226	0.222	0.209
Sample No. 2	0.210	0.215	0.197

Comments.—Here, as in the case of the F.E., the modification of the N. F. VIII method, using automatic extraction for the first series of alkaline-ether shake-outs, is superior to the present N. F. VIII method since it creates less manipulative difficulty.

Suggested Revision Method for the Assay of Tincture Ipecac, N. F.

Place exactly 50 cc. of Tincture Ipecac in a 250-cc. beaker and evaporate on a steam bath until the volume is reduced to 20 cc. Carefully transfer the 20 cc. to a Palkin–Watkins continuous extractor and rinse the beaker with two 20-cc. portions of distilled water, placing these portions in the extractor. Make the solution in the extractor distinctly alkaline with stronger ammonia water; carefully add the proper amount of peroxide-free ether. Connect the extractor to a condenser and extract for four hours. Occasionally disconnect the extractor from the condenser and agitate the lower layer by raising and lowering the center extraction tube. After the four-hour extraction period, disconnect the extractor and carefully transfer the ether to a 250-cc. separator. Wash the extraction flask with two small portions of ether and add these to the separator. Extract the alkaloids from the ether solution by shaking with four 25-cc. portions of 2% sulfuric acid. Now make the acid extractions alkaline by adding a slight excess of stronger ammonia and shake out with four 50-cc. portions of peroxide-free ether. Filter the ether through a filter paper into a 250-cc. beaker. Evaporate until the volume is about 2 cc., add 5 cc. alcohol, 20 cc. 0.05 *N* H_2SO_4, heat on the steam bath for a few minutes to expel the last traces of ether, then cool and titrate with 0.02 *N* NaOH, using Methyl Red as the indicator.

Each cc. 0.02 *N* H_2SO_4 = 4.80 mg. Ipecac alkaloids.

DISCUSSION

The over-all time factor in this revised method might possibly be a little longer in the Tincture assay than the present official method, but the actual man-time consumed will usually be less. Results obtained by using this method are slightly higher than those found in the official method for Tincture Ipecac.

The method suggested will give clean-cut separations with the aqueous and immiscible solvent phase, thereby eliminating the trouble usually encountered with emulsions.

In each case we have substituted automatic extraction for the first series of ether shake-outs. We believe this is advantageous since it is here that the alkaloids are separated from the other drug constituents and it is here, therefore, that complete extraction is essential, since manipulations by the official method are most difficult at this stage. We also believe that it is necessary to start out with a series of *alkaline ether extractions* in each case and not an acid dilution or acid filtrate. In this way, further purification of the alkaloids is obtained since the results in the acid filtrate method experiments are consistently higher, probably due to impurities present which a preliminary ether extraction would eliminate. We have successfully used the Soxhlet extraction method as suggested for the drug in analysis of Tablets of Ipecac and of an Ipecac Alkaloids Compound, which is in reality a concentrated mixture of the alkaloids absorbed on Lloyd's Reagent.

SUMMARY AND CONCLUSION

Various difficulties have been encountered in the analysis of Ipecac and its Fluidextract and Tincture by the official methods.

It is the desire of the authors of this paper to develop one general method that will apply not only to the Ipecac drug, but also to those pharmaceutical preparations which are made from Ipecac.

We have tried several modifications, in each case eliminating the methods which we found least desirable and finally emerging with methods which, by not too great a modification of the official methods, yield results which are more easily obtained and more readily reproducible.

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Influence of Preliminary Administration of Insulin or of Epinephrine Hydrochloride Upon the Fatal Dose of Sodium Evipal in Albino Mice* †

By HARALD G. O. HOLCK‡

Under conditions of this experiment using 104 mice, results show that neither epinephrine hydrochloride nor insulin, preliminarily administered, alter significantly the fatal dose of Sodium Evipal.

In 1945 Reinhard (1) reported that insulin given to white mice twenty minutes prior to Sodium Evipal prolonged the hypnosis induced by 100 mg. per Kg. of the latter drug by 67 to 89 per cent and that similarly administered epinephrine hydrochloride increased the sleeping time by 78 per cent.

In view of the increased use of intravenous anesthesia with ultrashort acting barbiturates and because of the common use of both insulin and of epinephrine, an experiment was undertaken to ascertain what effect, if any, doses similar to those employed by Reinhard would have upon the average fatal dose (LD_{50}) of Sodium Evipal in albino mice.

EXPERIMENTAL

The mice used were nonfasted male albino Swiss mice, weighing 18 to 28 Gm. The insulin was diluted with 0.9% sodium chloride solution so that 1 cc. contained one unit. One unit per Kg. of bodyweight was then administered into a lateral thigh muscle and twenty minutes later followed by Sodium Evipal given under the skin of the lower back; 0.91 unit per Kg. had previously been found to produce convulsions in 50% of similar mice under insulin assay conditions. The Sodium Evipal solu-

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‡ We are indebted to the Winthrop Chemical Company, Inc., for supplying the Sodium Evipal, and to Mr. David Russell for aid in the tests.

TABLE I.—COMPARISON OF SODIUM EVIPAL MORTALITIES IN MALE ALBINO MICE FOLLOWING MEDICATION WITH EPINEPHRINE HYDROCHLORIDE AND WITH INSULIN, RESPECTIVELY[a]

Sodium Evipal, Mg./Kg.	Mortalities		
		Pretreated with	
	Controls	Epinephrine	Insulin
375	1/13	0/13	1/13
447	4/13	1/13	1/13
533	3/13	1/13	2/13
635	3/13	5/13	5/13
757	6/13	8/13	11/13
903	8/13	10/13	13/13
986	10/13	9/13	13/13
1077	13/13	13/13	12/13
Total:	48/104	47/104	58/104
LD_{50} and S.E.,[b] mg./Kg.	730 ± 56	735 ± 45	640 ± 67

[a] Nonfasted male Swiss albino mice, injected subcutaneously with 5% solution of Sodium Evipal. One unit of insulin, or 0.04 mg. of epinephrine hydrochloride, or an equivalent amount of 0.9% sodium chloride solution per Kg. was injected intramuscularly 20 minutes prior to the barbiturate administration. The room temperature was 27 ± 1°.

[b] These estimates of the LD_{50} and their approximate standard errors were made by Dr. Lloyd C. Miller using a graphical procedure (3).

tion was 5% in distilled water and doses covered practically the whole fatal range. In case of epinephrine hydrochloride, 0.04 mg. per Kg. of mouse, using 2 cc. of a 50,000 dilution in 0.9% solution of sodium chloride was administered intramuscularly twenty minutes before Sodium Evipal. Although a considerable part of the epinephrine action may be over in twenty minutes, we chose the procedure of Reinhard (1), because his results indicated that with the large dose used—equivalent to 3 mg. in an adult person—the mice were clearly affected by epinephrine after twenty minutes as indicated by the prolonged hypnosis following Evipal administration. In case of the control mice, 1 cc. of 0.9% solution of sodium chloride per Kg. was similarly administered intramuscularly twenty minutes ahead of the Sodium Evipal. After medication the mice were kept individually in large glass beakers and the room temperature kept as close as possible to 27°. This is of particular importance because Chance (2) recently has reported that the toxicity of certain sympathomimetic amines, including epinephrine, was much greater in mice kept in groups after injection than in those kept in individual containers at our experimental temperature. The doses of Sodium Evipal, which started with 375 mg. per Kg., were increased in steps of 19.2%, except that in case of the dose of 986 mg. per Kg., the difference is only half the logarithmic increase, or 9.2%; this extra dose was employed to increase the number of animals in the range close to the surely fatal dose. A total of 104 mice were injected in each series and the results are presented in Table I.

Although our main concern was to study the influence of insulin and of epinephrine upon Evipal mortality, records were also kept of the recovery times until the mortality recording was discontinued at 10 p.m., after which the remaining mice were left for further observation on the following morning. Judging from the recovery times of about the first three-fourths of the mice only, we were unable to find any constant prolongation of depression due to insulin or to epinephrine in the fatal dosage range.

SUMMARY

Our results show that preliminary medication with a substantial dose of epinephrine hydrochloride did not influence the average fatal dose of Sodium Evipal when the latter was given twenty minutes later. Although the LD_{50} of Sodium Evipal is somewhat lower following medication with insulin, it is only 12.2 per cent below the control value; also, because the mortalities with the lower 4 or 5 doses are quite similar in the two cases and because the only mouse to survive 1077 mg./Kg. had been pretreated with insulin, it would appear that insulin does not significantly alter the fatal dose of Sodium Evipal under the conditions of our experiment.

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Note

Note on a Color Reaction for Shark-Liver Oil*

By LAWRENCE S. MALOWAN

For the purpose of detecting vitamin A in fish-liver oil by chemical means the reaction of Carr and Price (1) is the most generally used and is based upon the formation of a blue color when antimony trichloride is added to a solution of the vitamin-bearing oil. Recently proposed were glycerol 1,3-dichlorhydrin (2) and activated earth (3) as substitutes for antimony trichloride as colorimetric reagent.

EXPERIMENTAL

It was found that titanium tetrachloride in organic solvents produces a deep and stable color when added to a solution of shark-liver oil. Antimony trichloride produces no change when added to a solution of a fatty oil free from vitamin A or when added to solutions of fish-liver oil, the known vitamin A reaction. On the contrary, titanium tetrachloride in organic solvents mixed with fatty oils causes the formation of slightly red or brown colors. We investigated peanut, cottonseed, sesame, olive, castor, and linseed oils, all of which behave in the same way. Specific, however, is the reaction with shark-liver oil through the color effect shown.

Conduct of the Test.—If 1 ml. of a solution of 10% titanium tetrachloride in acetylene tetrachloride is mixed with 5 ml. of a 10% solution of shark-liver oil in chloroform, it causes a brilliant color display by producing at first a blue color which soon changes to a deep red violet. Even in presence of an excess of another fatty oil the reaction of shark-liver oil is distinctly visible, and the color does not fade for at least three hours.

DISCUSSION

Titanium tetrachloride in a pure state is not a very convenient reagent because of the dense fumes it produces, but it is easy to handle when dissolved in organic solvents, from which was chosen acetylene tetrachloride because of its high boiling point and the fact that, when mixed with the mentioned chloride, it does not produce any more fumes than hydrochloric acid. The reagent is quite stable; however, it contains some yellow precipitate.

* Received Nov. 6, 1947, from the Department of Biochemistry, University of Panama, R. P.

The mechanism of the reaction described was studied more closely by performing tests with the saponifiable and unsaponifiable fractions of shark-liver oil. The result has been that with neither fraction could be obtained any result identical to the effect produced by the shark-liver oil itself. The saponifiable fraction gives no specific reaction while the unsaponifiable portion produces a color-similar to that caused by antimony trichloride. Because of the formation of considerable precipitate the titanium reagent is unsuitable to be used for quantitative purposes. Carotene gives a blue color while cholesterol and phytosterol solutions do not show any change. Vitamin D in the form of viosterol or calciferol, however, gives a deep brown-red color with the titanium tetrachloride reagent, while antimony trichloride leaves the solution of vitamin D nearly colorless. By this factor the presence of vitamin D may be verified in medical preparations. Twenty-five tablets of Dical-D were extracted with chloroform for forty-eight hours. The extract was evaporated and the yellow residue redissolved in chloroform and mixed with the titanium tetrachloride solution. It developed a permanent deep red color. A similar experiment was carried out with Sterogyl; however, the vitamin solution in oil was primarily saponified. The unsaponifiable residue gave with the reagent a color similar to that obtained with the extract of Dical-D. The color developed by shark-liver oil with the titanium tetrachloride reagent is probably not due to its vitamin D content because it was negligible in the oils employed in these experiments.

SUMMARY

A simple color test for the detection of shark-liver oil, employing titanium tetrachloride, is described. This reagent is specific and allows to detect 0.5 ml. of the oil in presence of an excess of other fatty oils.

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